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Research Article

SIMVASTATIN-LOADED NANOCAPSULES REDUCE TNF-A EXPRESSION IN RAT PERITONEUM AFTER INFUSION OF PERITONEAL DIALYSIS SOLUTION

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ABSTRACT

Objective: Obteinment and characterizing polymeric nanocapsules of simvastatin (SV), and investigating their action in an experimental model of peritoneal fibrosis induced in a rat by the infusion of peritoneal dialysis (PD) solution.

Methods: Poly (ε-caprolactone) nanocapsules containing SV (NC-SV) were prepared by interfacial deposition of a preformed polymer. A suspension of nanoparticles with no drug was prepared as negative control. The average particle size and polydispersity index were measured by photon correlation spectroscopy. The morphological and surface evaluation of prepared nanocapsules was performed using field emission scanning electron microscopy. The ultra-high performance liquid chromatography with photodiode array detection method was used to evaluate the drug encapsulation efficiency. The release profiles of SV from polymeric nanocapsules were obtained by dialysis diffusion technique. The Animal Study was performed in a total of 48 male Wistar rats (*Rattus norvegicus*) divided in four groups: Sham, PD group, SV group, and Simvastatin-loaded nanocapsules group (NC-SV). After 28 days, tissue samples were surgically removed from the abdominal to perform histological and immunohistochemistry analysis. The statistical analysis was performed by one-way ANOVA followed by Bonferroni test, or by Kruskal–Wallis.

Results: NC-SV presented suitable particle parameters with a mean particle size of 332 nm, and an encapsulation efficiency of 99.87±0.46%. The expression of tumor necrosis factor-alpha (TNF-α) was significantly different in NC-SV group.

Conclusion: SV-loaded nanocapsules for controlled drug delivery were suitably prepared. This nanoformulation remarkable decreased the TNF-α tissue expression even at low SV dose in a chronic PD model.

Keywords: Chronic renal insufficiency, Encapsulating peritonitis, HMG-CoA reductase inhibitor, Interleukin-6, Nanotechnology, Peritoneal fibrosis, Tumor necrosis factor-α.

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INTRODUCTION

The prevalence of chronic kidney disease (CKD) is increasing worldwide and reached 2204 patients per million inhabitants in the USA. Peritoneal dialysis (PD) is a procedure used in 7.1% and 5% of patients on renal replacement therapy in the USA [1] and in Europe [2], respectively. The main obstacles to the long-term PD treatment are both infections and pathological changes in the peritoneal membrane exposed to dialysis solutions (DS), which affect 50% of PD patients. These patients may progress to the encapsulating peritonitis (EP) depending on the treatment time interval [3]. The risk of EP is significantly increased with PD treatment longer than 10 years (>10±12 years vs. >6±8 years, OR: 5.5). In addition, EP is a disease with high mortality, which reaches 74% in patients with severe forms [4]. It can also occur in children on PD, showing a similar prevalence to adults [5,6]. The symptoms onset may occur during PD. However, they may also occur very late, even after kidney transplantation [7,8]. In addition, when analyzing studies about the prevalence of EP, we have to take into account the diagnosis of this condition does not include a screening test to detect mild or asymptomatic cases, which explains the cases diagnosed years after the PD ending [9]. Therefore, the current prevalence of EP may be higher than that reported.

The pathophysiology of EP is complex. Factors that trigger the process are the exposure of peritoneal membrane to DS, bacterial infections, and inflammatory condition by uremia itself. Several mediators are involved, including cytokines [10]. Regardless of PD, uremia causes damage to the peritoneum. The carbonyl products formation occurs due to uremia, in addition to an accelerating production of advanced glycation end products. However, the PD onset also involves injury caused by catheter used and DS. This inflammatory condition could generate the peritoneum fibrosis and EP [11].

Simvastatin (SV) is a competitive reversible inhibitor of a natural substrate of hydroxymethylglutaryl coenzyme A reductase and the use of statins to prevent cardiovascular risk is well established. Its prescription depends on the analysis of cardiovascular risk of each patient. Patients with CKD are in the high-risk group; therefore, the prescription of statins is widely indicated to them [12]. Besides the lipid-lowering action, pleiotropic actions of statins have been described. These actions can contribute to a reduction of cardiovascular events in patients using statins. Statins inhibit the synthesis of isoprenoids, such as farnesyl pyrophosphate and geranyl pyrophosphate, also they inhibited the prenylation of specific proteins, such as Rho and Ras, which act through nuclear factor-κB (NF-κB), which is involved in the processes: Inflammation, proliferation and apoptosis, and it is implicated in atherogenesis. Statin treatment results in increased eNOS activity by reducing Rho prenylation. Statins also reduce the concentration of caveolin-1, which is the inhibitor partner of calmodulin and increases the affinity of binding of eNOS to calmodulin. Negative regulation of NF-κB pathway also leads to an increase in eNOS activity [13].

By these actions, statins have been used in several disease models, including their use in preventing peritoneal fibrosis. In a previous

study from our research group, Baroni *et al.* used the DS infusion model through daily puncture of the peritoneum to evaluate the influence of SV on fibrosis induced by DS in Wistar rats. Two groups of animals were subjected to a daily infusion of a 4.25% DS by intraperitoneal injection. One group received daily SV at a dose of 4 mg/kg, and the control group did not receive the drug. No effect was found on the use of SV, perhaps due to the low drug bioavailability [14].

Yeniçerioglu *et al*. studied the effect of atorvastatin in non-uremic Wistar rats. They were randomized in three groups: Sham (intraperitoneal saline), PD (PD 3.86% intraperitoneal dextrose-containing DS), and treatment (TX, 3.86% intraperitoneal dextrose containing DS plus atorvastatin added to drinking water at a dose of 80 mg/mL). The main findings were greater inflammation, fibrosis, and vascular proliferation in the PD group than in the Sham group. These features seemed to decrease significantly when atorvastatin was used as TX [15].

To evaluate the effect of SV on the mesenchymal epithelial transition action, Chang *et al*. carried one study in two parts. In vitro: Human peritoneal mesothelial cells (HPMCs) were exposed to 5.6 mmol/L glucose (NG) or 100 mmol/L glucose (HG) with or without SV at 1 mmol/L concentration. In vivo: 32 Sprague-Dawley rats were submitted to PD catheters with saline (C, n=16) or 4.25% PD fluid (PD, n=16) for 4 weeks. SV intraperitoneally treatment (5 mg/kg/day) were applied to eight rats from each group. E-cadherin expression presented a significant decrease when compared to NG cells, while fibronectin expression, a-SMA, Snail were significantly increased in HPMCs submitted to HG, all these changes were repealed by SV ($p<0.05$) [16].

Yang *et al.* carried out a study in rats, comparing a group which used SV (10 mol/L) with a group without SV, divided in a subgroup that used troglitazone (50 mol/L) and one that did not. Cholesterol and oxidized LDL (ox-LDL) content was measured in macrophages. The content of IL-6 and tumor necrosis factor-alpha (TNF-α) in macrophages was measured, as well as the expression of IL-6 and TNF-α mRNA. It was demonstrated that macrophages exposed to ox-LDL had an increase in cholesterol content and foam cells appeared, an effect normally reversed by use of SV. The use of troglitazone abolished this effect [17]. Kim *et al*. showed that the presence of SV reduces the expression of IL-6 and IL-8 in an *in vitro* study, as well as reduces the expression of reactive oxygen species in cells isolated from intestinal cells stimulated by TNF-α. The same group, in a study model of intestinal ulceration *in vivo*, inducing lesions by anti-inflammatory drugs, demonstrated that the use of SV reduced the expression of reactive oxygen species, as well as the number of intestinal ulcerations [18].

None of papers briefly mentioned above used nanotechnology to optimize the drug effect. At present, one of the biggest challenges of the pharmaceutical industry is developing drugs with maximum of their therapeutic effect and reduction of their adverse effects [19], since approximately 40% of the discovered substances are left aside, mainly because of their low solubility and bioavailability [20]. To overcome these problems, the use of nanotechnology has altered the panorama of the pharmaceutical industries by enabling the development of safer and more effective drugs, with better biocompatibility and taking advantage of all their action potential [21].

SV is a lactone prodrug, which is modified in the liver. SV is a white, crystalline, and non-hygroscopic powder with log p=4.4. It is practically insoluble in water (30 μg/mL) and 0.1 mol/L HCl (60 μg/mL). Due to its very low aqueous solubility, SV exhibits controlled absorption by dissolution and, therefore, shows reduced absorption, distribution and arrival at the target organ. Various procedures can be used to improve SV solubility by enhancing its bioavailability as inclusion complex, solid dispersion, surfactant solubilization, and particle reduction techniques [22]. Therefore, nanotechnology can significantly improve the drug effects by creating new SV-loaded formulations.

The objective of this study was to develop and characterize SV-loaded nanocapsules and compare with pure drug and its effect on peritoneal fibrosis and on expression of TNF-α and IL-6 induced by the use of 4.25% DS.

METHODS

Preparation of polymeric nanocapsules

Poly (ε-caprolactone) (PCL) nanocapsules containing simvastatin (NC-SV) were prepared by interfacial deposition of a preformed polymer [23]. Briefly, 100 mg of PCL (Mw 14,000 g/mol, Sigma-Aldrich, St. Louis, MO, USA) was solvated in acetone (27 mL) in the presence of sorbitan monostearate (Span® 60, 77 mg), triglycerides of capric/caprylic acids (MCT, 300 mg), and SV (SV, 200 mg, Mw 418.57 g/mol, lot DK40-1506061, certified by Galena Farmacêutica, Campinas, Brazil). This organic phase was then dripped slowly into the aqueous phase containing water (53 mL) and polysorbate 80 (Tween® 80, 77 mg), previously prepared and maintained under vigorous magnetic stirring at 40°C. The magnetic stirring was kept for another 10 min after the end of the dripping and the solvent was eliminated using a rotary evaporator (FISATOM, model 801, Diadema, Brazil), reaching a final volume of 10 mL and drug concentration of 20 mg/mL. This formulation was obtained in triplicate from three different batches. A suspension of nanoparticles with no drug (NC-N) was prepared as negative control.

Characterization of polymeric nanocapsules

The average particle size and polydispersity index (PDI) (n=3) were measured by photon correlation spectroscopy (Zetasizer Nanoseries, Malvern Instruments, Malvern, UK) after diluting each sample in ultrapure water (1:500, v/v). At the same equipment and using the same sample preparation, zeta potential (n=3) was determined by electrophoretic mobility technique.

The morphological and surface evaluation of prepared nanocapsules was performed using field emission scanning electron microscopy (FESEM) (Tescan, model Mira 3, Brno, Czech Republic). The samples were previously submitted to metallization with gold in an IC-50 Ion Coater metallizer (Shimadzu, Kyoto, Japan). An acceleration voltage of 8–10 kV was used to obtain electromicrographs.

The ultra-high performance liquid chromatography with photodiode array detection (UHPLC-DAD) method was used for evaluate the drug encapsulation efficiency using a previously validated analytical method [24] with modifications. In brief, suspension of nanocapsules was submitted to a combined ultrafiltration/centrifugation using centrifugal devices (Amicon® 10,000 NMWL, Millipore, Bedford, MA, USA) at 2200 g during 30 min in triplicate. Free SV was determined in ultrafiltrate using an UHPLC method in Nexera X2 equipment (Shimadzu, Kyoto, Japan). The UHPLC system was equipped with a column oven compartment (model CTO-20AC), an on-line degasser (model DGU-20A5RA), a quaternary pump (model LC-30AD), an auto sampler (model SIL-30 AC), a communication bus module (model CBM-20A), and a photodiode array wavelength detector (model SPD-M20A). Data acquisition, analysis, and reporting were performed using LabSolutions® chromatography software (Shimadzu, Milton Keynes, UK, version 5.73). Chromatographic separation was accomplished using a Shimadzu Shim-pack XR-ODS III C18 analytical column (Kyoto, Japan) with 2.2 μm particle size, 2.0 mm internal diameter at temperature of 40±2°C using UV detection at 237 nm. The isocratic mobile phase consisted of acetonitrile: water acidified with phosphoric acid (83:17, v/v) at a flow rate of 0.75 mL/min. The encapsulation efficiency (EE, %) was calculated using Eq. (1).

$$EE(\%)=\frac{\text{Theoreticaldrugloading}-\text{Freedrugcontent}}{\text{Theoreticaldrugloading}}\times 100 \quad (1)$$

In vitro drug release study

The release profiles of SV from polymeric nanocapsules were obtained by dialysis diffusion technique. Each sample (30% ethanol-water solution of SV and NC-SV) was placed into a dialysis bag (Spectra/Por® molecular porous membrane tubing, MWCO 10,000, Spectrum

Laboratories, Rancho Dominguez, CA, USA) at an equivalent concentration of 1 mg/mL and a final volume of 3 mL. This system was then immersed in 500 mL of water with 30% of ethanol at 37°C and kept under continuous magnetic stirring of 50 rpm. Aliquots of 5 mL were withdrawn at predetermined time intervals and replaced by the same volume of fresh medium. The amount of SV released was assayed by the aforementioned UHPLC-DAD method. The experiment was performed in triplicate from three independent batches.

Animal study

The *in vivo* study was submitted to the Animal Ethics Committee from State University of Ponta Grossa (UEPG) and approved under number 37/2018. A total of 48 male Wistar rats (*Rattus norvegicus*) weighting from 200 to 250 g were obtained from the Animal House Facility at the UEPG, Faculty of Medicine. The animals were fed a standard rat pellet diet and it was allowed free access to water. The rats were kept in labeled cages with a maximum of five animals per cage at 25±2°C and 12-h light/dark cycle.

The intraperitoneal injection model was performed for induction of peritoneal fibrosis. A DS with glucose at 4.25% buffered with lactate (Baxter Hospitalar, São Paulo, Brazil) was intraperitoneally injected using a 25 × 7/8" needle. There were four experimental groups, divided as follows: Group 1 (Sham), 12 animals submitted to the daily repeated puncture for 28 days with no solution infusion; Group 2 (PD group), 12 animals subjected to the daily peritoneal puncture for 28 days with DS infusion at a dose of 10 mL/100 g of weight; and Group 3 (SV group), 12 animals submitted to the daily repeated puncture for 28 days with DS infusion at 10 mL/100 g of animal weight. This group also received 4 mg/kg of SV per gavage daily during 28 days. Group 4 (SV-loaded nanocapsules group – NC-SV), 12 animals submitted to the daily peritoneal puncture for 28 days with DS infusion at 10 mL/100 g of weight. This group received NC-SV containing 4 mg/kg of SV per gavage daily.

After 28 days, the animals were slaughtered and 1-cm^2 tissue samples were surgically removed from the abdominal wall of the upper right quadrant as far as possible from the puncture point. After the routine paraffin embedding, 4-µm thick sections were stained with hematoxylin and eosin and Sirius red (SR). Ten histological fields were chosen and ten peritoneum thickness measurements were taken in each field using the Axio Scan.Z1 digital slide scanner (Carl Zeiss Microscopy, Jena, Germany). The amount of collagen types I and III, in percentage per high power field (HPF), was automatically measured using SR staining with the Axio Scope.A1 polarized light microscope (Carl Zeiss Microscopy, Jena, Germany).

For immunohistochemistry, the 4-µm thick sections were rehydrated in decreasing ethanol concentration and incubated in 5% hydrogen peroxide and methanol. These samples were then immersed in ImmunnoRetrivier® (Dako, Carpinteria, CA, USA), followed by incubation in diluted solution of antibodies anti-TNF-α (ab6671, 1:100, Abcam®, Cambridge, UK) and anti-IL-6 (ab9324, 1:400, Abcam®, Cambridge, UK) at 4ºC. Then, the reaction was carried out using 3-3'-diaminobenzidine chromogen solution (code D7679, Sigma-Aldrich, St. Louis, MO, USA), followed by the staining with Harris' hematoxylin solution (Biotec, Lages, Brazil), the ethanol dehydration process, and the clearing using xylene. The slides were observed using the Axio Scan.Z1 digital slide scanner and the ZEN 2.3 program (Carl Zeiss Microscopy, Göttingen, Germany) concerning to the presence/absence of TNF-α and IL-6inpercentage per HPF of peritoneal samples.

The statistical analysis was performed by one-way ANOVA, followed by Bonferroni test, except for results of TNF-α, in which the Kruskal–Wallis test was used. $p<0.05$ was considered statistically significant.

RESULTS

Preparation and characterization of polymeric nanocapsules

SV-loaded PCL nanocapsules (NC-SV) and non-loaded PCL nanocapsules (NC-N) suspensions showed a uniform milky white appearance and atypical opalescent bluish reflection. NC-SV presented suitable particle parameters for oral use as mean particle size of 332 nm, PDI of 0.290, and zeta potential of –58.7 mV.

Nanoscale dimensions were registered for NC-SV (a) and NC-N (b) when their images were assessed by FESEM (Fig. 1). NCs were spherically shaped and had smooth surface with uniform distribution. In addition, no drug crystals were seen on their surfaces.

The proposed UHPLC-DAD method was used for determining the drug-loading efficiency. Fig. 2 depicts the chromatograms obtained for SV-loaded (a) and non-loaded (b) nanocapsules. SV showed an appropriate separation at short retention time of 1.71 min.

Comparing both chromatograms, the other formulation components demonstrated no interference in drug quantification. Considering all the chromatographic data, a high encapsulation efficiency of 99.87±0.46% was achieved for NC-SV.

***In vitro* drug release study**

The SV release profiles as pure drug and nanoformulation (NC-SV) are reported in Fig. 3. The free SV showed a rapid drug release profile by reaching a mean release value of 80% after 148 min of the experiment.

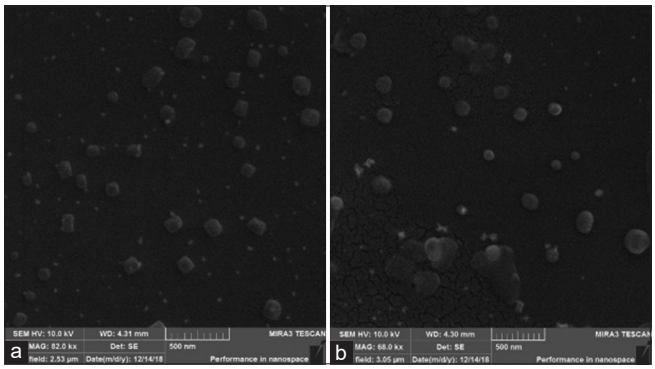

Fig. 1: Photomicrographs of simvastatin-loaded and non-loaded nanocapsules observed by FESEM: (a) NC-SV (68 k× magnification) and (b) NC-N (82 k× magnification)

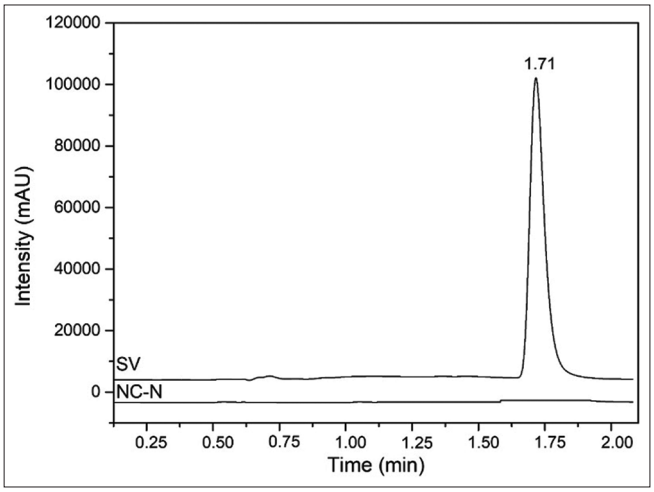

Fig. 2: UHPLC-DAD chromatograms obtained for simvastatin-loaded (a) and non-loaded (b) nanocapsules. Chromatographic conditions:isocratic elution mode; mobile phase: acetonitrile:water acidified with phosphoric acid (pH 3.0) (83:17, v/v); flow rate: 0.75 mL/min; UV detection wavelength: 237 nm; column temperature: 40±2°C; run time: 2.11 min; and retention time: 1.71 min

The NC-SV formulation released 80% SV at 2880 min (48 h). Therefore, the SV-loaded nanocapsules demonstrated a prolonged release profile with no burst effect, when compared with pure drug.

Animal study

The results of histological analysis are summarized in Table 1. In brief, no statistically significant difference was observed for peritoneal thickness when experimental groups were compared. However, a significant increase in the inflammation-associated collagen type III was achieved for PD group ($p<0.001$) when compared with Sham group. The SV group also presented a statistical difference in collagen type III expression comparing with Sham group ($p<0.001$), while the effect of NC-SV concerning to collagen type III was statistically similar ($p>0.05$) to the Sham group. The nanoformulation also revealed a statistical difference in collagen type III when compared with other experimental groups, PD group ($p<0.001$) and SV group ($p<0.05$). The same statistical significance was obtained to a usual collagen type I considering the studied groups due to its complementary nature in total collagen amount to reach 100%.

The results of TNF-α tissue expression in the peritoneal samples are depicted in Fig. 4. The TNF-α tissue expression was 23.5±14.5%, 20.3±11.1%, and 0.79±0.76% (HPF) for PD and SV an NC-SV groups, respectively.

No statistical difference was observed between PD and SV groups. However, a statistically significant decrease in TNF-α tissue expression was achieved when NC-SV group was compared with PD ($p<0.0002$) and SV ($p<0.0002$) groups.

The immunohistochemical images for TNF-α expression in the peritoneal tissue of rats are shown in Fig. 5. TNF-α immunoslides indicated that the TNF-α protein expression (%/HPF) was significantly higher in the peritoneal section obtained from PD and SV groups (Fig. 5a and b). Nanoformulation treatment containing simvastatin (NC-SV group) prevented the increase in peritoneal tissue expression of TNF-α (Fig. 5c).

Concerting to the IL-6, the tissue expression was 3.14±1.35%, 2.72±1.28%, and 2.62±1.32% (HPF) for PD, SV, and NC-SV groups, respectively. No statistically significant difference was observed between paired groups.

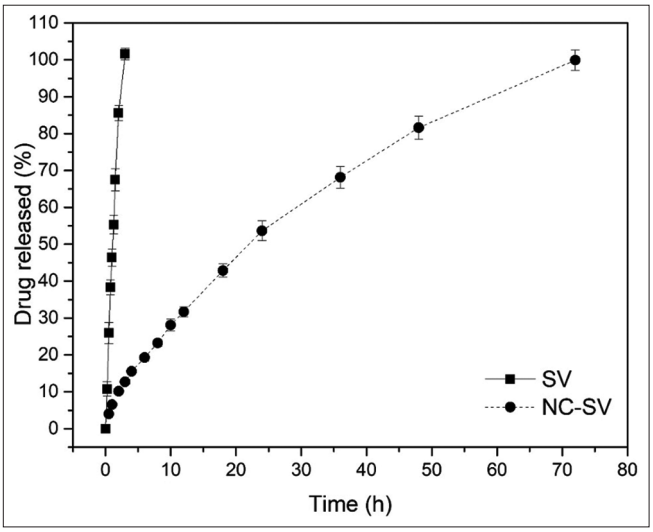

Fig. 3: In vitro drug release profiles of free simvastatin (SV) and simvastatin-loaded nanocapsules (NC-SV) using 30% ethanol-water solution as dissolution medium

Table 1: Peritoneum thickness and collagen amount in the experimental groups submitted to the chronic use of 4.25% DS for 28 days

Parameters	Experimental groups			
	SHAM	PD	SV	NC-SV
Thickness* (μm)	19.1±6.0	20.1±4.9	19.2±6.1	25.5±9.1
Collagen type I* (%/HPF)	82.9±7.8	77.2±12.4	56.8±13.6	79.6±8.1
Collagen type III* (%/HPF)	17.1±7.8	22.8±12.4	43.2±13.6	20.4±8.1
Collagen ratio type I: III	4.85:1	3.38:1	1.31:1	3.96:1
Increase in collagen type III expression**	–	↑1.33 fold (+33%)	↑2.53 fold (+153%)	↑1.19 fold (+19%)

*Values are depicted as mean±standard deviation, **Significant differences occur in the Collagen I: III ratio in Group SV when compared to the others. DS: Dialysis solutions, PD: Peritoneal dialysis, SV: Simvastatin, NC-SV: Nanocapsules containing simvastatin, HPF: High power field

DISCUSSION

In this study, SV-loaded nanocapsules were successfully obtained as nanosuspensions by the interfacial deposition of preformed polymer. This formulation presented a final opalescent bluish aspect due to the Tyndall effect from colloids [23,25]. The mean particle size and PDI value were similar to those previously recorded by photon correlation spectroscopy for other polymeric nanocapsules [26]. PDI values close to zero are considered monodisperse and >0.5 indicate heterogeneous dispersion [23]. Zeta potential was also suitable since it was higher than |30 mV|, which is usually considered having sufficient repulsive force to achieve better physical colloidal stability [25]. The electron micrographs confirmed the spherical shape and the smooth surface, which are typical to polymeric nanocapsules, obtained using PCL as polymer [27]. Hence, the nanoformulation containing SV showed all required features in terms of size, superficial charge, and morphology for its use by oral, intraperitoneal, and intravenous routes.

However, it is essential to investigate the encapsulation efficiency to discover the total drug entrapped within the colloidal nanoformulation after its preparation and to use corresponding drug amount in animal studies. SV-loaded PCL nanocapsules (NC-SV) had encapsulation efficiency near to 100%. This result is better than that one made by Gambhire *et al*., who obtained SV-loaded solid lipid nanoparticles by pre-emulsion followed by the ultrasonication with an encapsulation efficiency of 72.52% [28]. It was also slightly better than the result achieved by Zhang *et al*., who prepared lipid nanoparticles by emulsion-solvent evaporation and achieved encapsulation efficiencies from 97.2% to 99.2% [29]. Shinde and More studied poly (lactic-*co*-glycolic acid) nanoparticles obtained by nanoprecipitation and recorded encapsulation efficiencies between 87.03% and 97.18% [30]. These high values of encapsulation efficiency are mainly due to the poor aqueous solubility of SV, which provides a high drug entrapment in oil/lipid-based formulations [29].

The next stage was to investigate the *in vitro* drug release in order to confirm if nanoformulation could provide a controlled-release pattern. A typical structure of an oily core surrounded by a polymeric shell was strategically planned for this purpose to avoid the immediate release of SV and to obtain a prolonged response. This approach was crucial to avoid the administration of multiple doses per day on rats and ensuring a longer-lasting pharmacological effect than free drug. Our nanoformulation containing SV proved a prolonged release of the drug during 48 h with no relevant initial burst. Fathi *et al*. [31] prepared lipid nanoparticles through emulsification-solvent evaporation followed by ultrasonication and reported an initial rapid release of SV using dialysis-spectrophotometry, which was proportional to the oleic acid and the surfactant content, followed by a prolonged release for 24 h. Therefore, the formulation NC-SV can provide a controlled-release pattern for SV

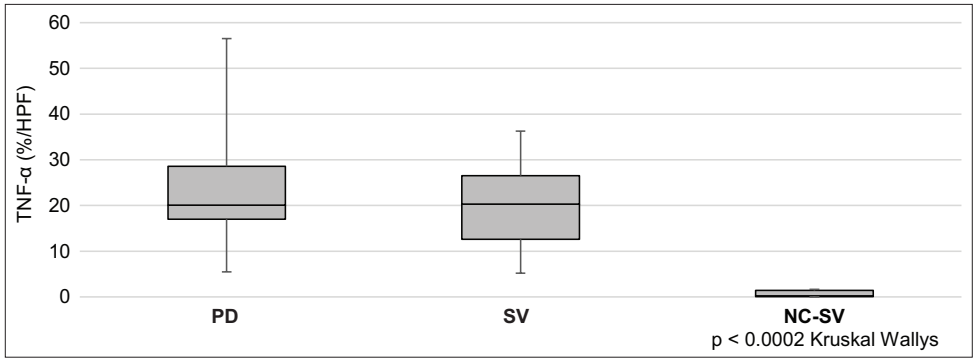

Fig. 4: Tumor necrosis factor-alpha (TNF-α) tissue expression (percentage per high power field) in the peritoneal samples of rats treated with peritoneal dialysis solutions (PD), simvastatin (SV), and simvastatin-loaded nanocapsules (NC-SV)

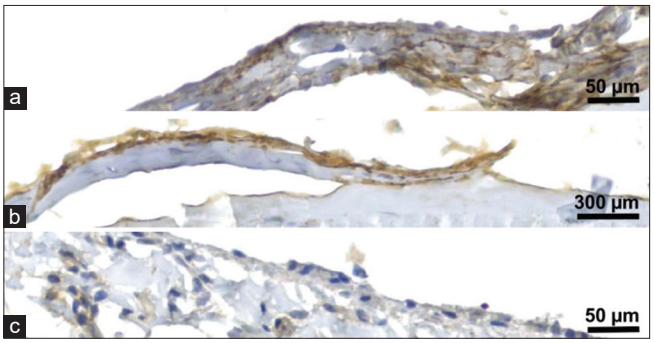

Fig. 5: Results of tumor necrosis factor-alpha (TNF-α) immunoexpression in the experimental groups, where the brown precipitate indicates the presence of the target antigen. TNF-α level was high in the peritoneal tissue slices of peritoneal dialysis rats (a), markedly present in the peritoneal tissue of simvastatin group (b), but not in rats treated with NC-SV (c). Several microphotographs were taken using a ×10–100 magnification objective and the images are representative of the experimental results

and can ensure a better pharmacokinetics since this nanocarrier can also avoid the SV extensive metabolism by cytochrome-3A system in gut and liver [29].

Regarding to the animal study, SV and NC-SV groups did not decrease the peritoneum thickness as expected. These results may be associated with the low dose of SV used (only 4 mg/kg) as free drug and in the SV-loaded nanocapsules. Rats can support very high doses of SV [32]. Besides that, a previous animal study involving the administration of SV at 25 mg/kg did not observe changes in bone tissue, after ovariectomy, or in bone healing of rats [33]. However, the use of atorvastatin added into drinking water at a dose of 80 mg/mL provided significant minor fibrosis and thinner peritoneum [15]. In general, Wistar rats usually drink 20–50 mL water a day (10 mL/100 g body weight/day) [34]. Hence, high doses of atorvastatin reaching above 1.6 g/day were used for achieving morphological changes in peritoneum thickness of rats. In this sense, we assume that low dose of SV used was accountable for the lack of effects on the peritoneal thickness of Wistar rats in the SV and NC-SV groups.

When the type of collagen was evaluated, the NC-SV group showed an expression of type III collagen statistically similar to sham and DP. Therefore, nanoformulation was able to reduce type III collagen associated with inflammation in order to control levels. Type III collagen is secreted by fibroblasts and other types of mesenchymal cells, making it an important element in several diseases associated with inflammation, such as renal fibrosis [35]. Consequently, the low expression of type III collagen provides strong evidence that inflammation has been significantly suppressed by nanoformulation, which was not so remarkable with the use of free SV, although the anti-inflammatory effect of SV is well known to inhibit formation of intracellular isoprenoids and improve the bioavailability of NO [12].

The reason of DP group was not significantly different from the sham which is not very clear. One possible explanation is due to the different manipulation between these groups, since the sham group was not subjected to gavage. It is known that damage to the esophageal mucosa caused by gavage can alter the cells of intestinal epithelium. The damage of esophageal epithelium causes a reduction in γδ T cells, which, in turn, plays an important regulatory role in maintaining the function of intestinal epithelium barrier and its cell renewal. The reduction in γδ T cells can also cause a reduction in CD3+ cells. This study provides initial evidence of the importance of esophageal integrity and intestinal cell populations and that the use of prolonged and repeated gavage can be accompanied by immunological complications [36].

Recently, it has been discovered that not only do these γδ T cells provide essential epithelial growth factors, which are crucial for maintaining tissue homeostasis and timely return to steady-state after damage, but they also act quickly to compartmentalize and limit microbial pathogenic exposure to the systemic immune compartment [37]. We can hypothesize that animals submitted to repeated gavage may have had a more intense inflammatory reaction. Anyway, in the NC-SV group, which was also subjected to gavage, the pattern of proportion between types I and III collagen was similar to that of sham. This could be explained by the more intense anti-inflammatory action of this formulation.

Considering the promising results obtained for the NC-SV group during the collagen analysis, the investigation was deepened to verify which inflammatory cytokines was inhibited. The nanoformulation decreased expressively the TNF-α level expression in peritoneal tissue of rats, what was not observed for free SV. TNF-α plays a key role in many inflammatory diseases. It is produced by macrophages, natural killer cells, neutrophils, and several non-hematopoietic cells. Although TNF-α was initially identified by its cytotoxicity against certain cell lines, its primary function (associated with IL-1) is to initiate the cascade of factors associated with inflammatory response. Therefore, it seems like the nanoformulation was able to improve the drug amount in peritoneal tissue and also ensuring the suitable anti-inflammatory effect of SV at low dose against the inflammatory status provided by the PD procedure. Some previous reports demonstrated the higher intestinal permeability and the increased bioavailability when nanosystems were used as technological approaches for SV [31,38]. Moreover, a significant reduction in inflammatory cytokines, including TNF-α and IL-6, was observed in other animal study involving intestinal ischemia in rats and SV at a dose of 20 mg/kg [39]. In that sense, the anti-inflammatory effect provided by the NC-SV can be associated with an increase of collagen type III expression and a significant reduction

of TNF-α expression when the experimental rat model of peritoneal fibrosis induced by infusion of PD solution was used.

Considering the reduction of IL-6 by SV in vascular smooth muscle cells and macrophages was previously reported [37], this cytokine expression was also investigated. However, IL-6 levels were similar among the PD, SV, and NC-SV groups. Kishimoto [41] proposed that IL-6 is a multifunctional cytokine involved in cell proliferation and differentiation, maintaining immune homeostasis, macrophage function, and other key functions. IL-6, acting through the latent transcription factors signal transducer and activator of transcription-3 (STAT3) and STAT1, also plays pivotal roles in governing leukocyte infiltration during acute inflammation [42]. In this present study, no significant change in the IL-6 tissue expression was found for pure SV and SV-loaded nanoformulation. The possible reason may be that IL-6 is an acute pro-inflammatory mediator and the animal model involved the chronic and long-lasting use of 4.25% DS. In general, the detection of IL-6 occurs in studies performed during few days [40], but this inflammatory stimulus was kept for almost 1 month in the present study.

CONCLUSION

SV-loaded nanocapsules for controlled drug delivery were suitably prepared. This nanoformulation remarkable decrease the inflammation-associated collagen type III and the TNF-α tissue expression even at low SV dose in a chronic PD model and may be further investigated as a nanotechnology-based approach for treating peritoneal fibrosis or even EP.

We can also corroborate with Alston *et al.* considerations that EP is a condition related to several pathological processes that lead to the appearance of adhesion, fibrosis, vasculopathy, and the formation of encapsulated tissue on the injured peritoneal surface [43]. EP can represent a multidimensional pathological process and novel therapeutic approaches need to be proposed.

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