Zein based-Nanoparticles Improve the Oral Bioavailability of Resveratrol and its Anti-inflammatory Effects in a Mouse model of Endotoxic Shock

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Running title: Zein nanoparticles improve bioavailability and antiinflammatory effect of resveratrol

Abstract

Resveratrol offers pleiotropic health beneficial effects including its reported capability to inhibit lipopolysaccharide (LPS) induced cytokine production. The aim of this work was to prepare, characterize and evaluate a resveratrol nanoparticulate formulation based on zein. For this purpose the oral bioavailability of the encapsulated polyphenol as well as its anti-inflammatory effect in a mouse model of endotoxic shock were studied.

Resveratrol-loaded nanoparticles displayed sizes around 300 nm with a negative zeta potential (- 51 mV) and a polyphenol loading close to 80 µg/mg. In vitro, the release of resveratrol from the nanoparticles was found to be pH-independent and adjusted well to the Peppas-Salin kinetic model, suggesting a mechanism based on the combination between diffusion and erosion of the nanoparticle matrix. Pharmacokinetic studies demonstrated that zein-based nanoparticles provided high and prolonged plasma levels of the polyphenol for at least 48 h. The oral bioavailability of resveratrol when administered in these nanoparticles increased up to 50% (20-fold higher than for the control solution of the polyphenol). Furthermore, nanoparticles administered daily for 7 days at 15 mg/kg, were able to diminish the endotoxic symptoms induced in mouse by the ip administration of LPS (i.e. hypothermia, piloerection and stillness). In addition, serum TNF-α levels were slightly lower (about 15%) of those observed for the control.

Key words

Resveratrol, zein, nanoparticles, bioavailability, anti-inflammatory.

Abbreviations

Rsv: resveratrol
SIRT1: sirtuin 1
LPS: lipopolysaccharide from Salmonella enterica serovar. Minnesota
Rsv-NP-Z: resveratrol-loaded zein nanoparticles
NP-Z: empty zein nanoparticles
Rsv-sol: resveratrol solution in a PEG 400: water mixture
Rsv-susp: suspension of resveratrol in purified water
PCS: photon correlation spectroscopy
SEM: Scanning electron microscopy
EE: encapsulation efficiency
iv: intravenous
C_{max}: maximal serum concentration
T_{max}: time in which Cmax is reached
AUC: area under the concentration-time curve from time 0 to last time
MRT: mean residence time
Cl: clearance
V: volume of distribution
$t_{1/2}$: half-life in the terminal phase
Fr: relative bioavailability
FRD: fraction of resveratrol dissolved
FRA: fraction of resveratrol absorbed
ip: intraperitoneal
PGE2: prostaglandin E2
PDI: polydispersity index

Introduction

Resveratrol (Rsv) (3,5,4'-trihydroxy-trans-stilbene), is a polyphenol molecule that was identified from the dried roots of *Polygonum cuspidatum*, a plant used in traditional Chinese and Japanese medicine [1]. Resveratrol has been classified as a phytoalexin as it is synthesized in spermatophytes in response to injury, UV irradiation and fungal attack [2]. It is naturally found in a wide variety of plant species, vegetables, fruits and food products such as peanuts, grape skin, plums or red wine [2].

Resveratrol offers pleiotropic health beneficial effects, including antioxidant and anti-aging effects [3], cardioprotective [4], anticancer [1], neuroprotective [5] and HIV/AIDS activities [6].

In the lasts years, it has been demonstrated the preventive effect of resveratrol against diabetes. Resveratrol would normalize hyperglycaemia and, in animals with hyperinsulinemia, it would reduce blood insulin [7]. Similarly, resveratrol was reported to reduce body weight and adiposity in obese recipients [8]. These actions would involve the activation of sirtuin 1 (SIRT1) that inhibits inflammatory pathways in macrophages and modulates insulin sensitivity [9]. Furthermore, different studies have shown that resveratrol is capable of inhibiting lipopolysaccharide (LPS) induced cytokine production [10]. This effect, via modulation of NF-κB, would decrease the production and gene expression of IL1 and TNF-α, important endogenous pyrogens [11].

In spite of these potential health benefits, the use of resveratrol is limited due to its high lipohilicity, short biological half-life, and chemical instability. In addition, when resveratrol is orally administered, only trace amounts of the unchanged polyphenol can be detected in plasma [12]. This low bioavailability is due to the polyphenol biotransformation by UDP-glucuronosyltransferase and sulphotransferases that produces resveratrol-3′-glucuronide and the sulphate derivative, respectively [2]. In rats, the main metabolite of resveratrol is the glucuronide conjugate [13], whereas, in humans, both the glucuronide and the sulphate derivatives have been described [14]. These metabolites have a longer plasma half-life, however, their efficacy are unknown [1].

Renal excretion is the major route of elimination of the polyphenol and its derivatives [2,15].

In order to solve these drawbacks different strategies have been pursued including its encapsulation in different oral delivery systems such as, among others, self-nano emulsifying drug delivery systems [16], solid lipid nanoparticles [17] and polymeric nanoparticles [18].

An alternative approach might be the use of zein nanoparticles. Zein is the major storage protein of maize and comprises aprox. 45-50% of the total protein content in corn [19]. Since zein is a natural protein, it is actually a heterogeneous mixture of different peptides than can be divided in four main fractions: i) α-zein (75-85% of total protein) with two main MW of 21-25 kDa and 10kDa, ii) β-zein (10-15%) of a MW of 17-18 kDa, iii) δ-zein, a minor fraction of 10kDa and vi) γ-zein (5-10%) with a MW of 27 kDa [19,20]. Zein is an amphiphilic protein, possessing high percentages of hydrophobic amino acids such as leucine (20%), proline (10%) and alanine (10%) [19,20]. Due to this amino acid composition, zein is insoluble in water and, thus, the resulting devices (e.g. films, nanoparticles) display an hydrophobic character with interesting properties to control the release of the loaded compound [20,21]. In addition, as for other nanocarriers from protein origin, they are biodegradable and can accommodate a great variety of compounds in a non-specific way [22].

Therefore, the aim of this work was to prepare, characterize and evaluate a resveratrol nanoparticulate formulation based on zein and to study its oral bioavailability and anti-inflammatory effect in a mouse model of induced endotoxic shock.

Material and Methods

Chemicals

Zein, resveratrol, lysine, mannitol, sodium ascorbate, poly(ethylene glycol) 400 (PEG 400) and Tween 20 were purchased from Sigma-Aldrich (Germany). Resveratrol-3-O-

131 D-glucuronide (Rsv-O-glu) was from @rtMolecule (Poitiers, France). Ethanol,
132 methanol, acetic acid and acetonitrile HLPC grade were obtained from Merck
133 (Darmastadt, Germany). Lipopolysaccharide from *Salmonella enterica* serovar.
134 Minnesota (LPS) was purchased from Sigma®, (St. Louis, USA). Deionised reagent
135 water (18.2 MO resistivity) was prepared using a water purification system (Wasserlab,
136 Spain). All reagents and chemicals used were of analytical grade.

137 **Preparation of resveratrol-loaded nanoparticles (Rsv-NP-Z)**

138 Nanoparticles were prepared by a desolvation method followed by an ultrafiltration
139 purification step and subsequent drying in a spray-drier apparatus. Briefly, 600 mg zein
140 and 100 mg lysine were dissolved in 60 mL of an ethanol:water mixture (65% ethanol
141 by vol.). In parallel, 100 mg resveratrol were dissolved in 10 mL ethanol and 6 mL of
142 this solution were transferred to the zein solution. In addition, 6 mg sodium ascorbate
143 were added to minimise the oxidation of the polyphenol. The mixture was magnetically
144 stirred in the dark for 10 min at room temperature. Nanoparticles were obtained by the
145 continuous addition of 60 mL of purified water. The suspension was purified and
146 concentrated by ultrafiltration using a 50 kDa pore size polysulfone membrane
147 cartridge (Medica SPA, Italy). Then, 15 mL of purified water containing 1.2 g mannitol
148 were added to the resulting suspension of nanoparticles to prevent aggregation and
149 irreversible interactions among nanoparticles during the drying process. Finally the
150 suspension was dried in a Büchi Mini Spray Drier B-290 apparatus (Büchi Labortechnik
151 AG, Switzerland) under the following experimental conditions: (i) inlet temperature: 90
152 °C, (ii) outlet temperature: 45-50 °C, (iii) air pressure: 4-6 bar, (iv) pumping rate: 5
153 mL/min, (v) aspirator: 100% and (vi) air flow: 400-500 L/h.
154 Control formulations (NP-Z) were prepared as described above but in absence of
155 resveratrol.

156 **Preparation of resveratrol conventional formulations**

157 Two different formulations of resveratrol were also prepared. The first one, a solution of
158 the polyphenol in a mixture of PEG400 and water (1:1 by vol.) was preparing dissolving

37.5 mg of resveratrol in 5 mL of PEG400 under magnetic stirring. Then 5 mL of purified water were added and the final mixture was agitated in the dark for 10 min. This formulation was named Rsv-sol.

The second one was an extemporary suspension of resveratrol in purified water (Rsv-susp). Briefly, 37.5 mg of resveratrol were dispersed in 10 mL of purified water under magnetic agitation for 10 min. The size of the resulting suspension was 21.4 ± 9.2 µm. The suspension was used after inspection for absence of aggregates.

Characterization of nanoparticles

Size, zeta potential and morphology

The mean hydrodynamic diameter and the zeta potential of nanoparticles were determined by photon correlation spectroscopy (PCS) and electrophoretic laser Doppler anemometry, respectively, using a Zetamaster analyzer system (Malvern Instruments Ltd., Worcestershire, UK). The diameter of the nanoparticles was determined after dispersion in ultrapure water (1:10) and measured at 25 ºC with a scattering angle of 90 °C. The zeta potential was measured after dispersion of the dried nanoparticles in 1 mM KCl solution.

The morphology of the nanoparticles was studied using a field emission scanning electron microscopy (SEM) in a Zeiss DSM940 digital scanning electron microscope (Oberkochen, Germany) coupled with a digital image system (Point Electronic GmBh, Germany). The yield of the process was calculated by gravimetry as described previously [22].

Resveratrol analysis

The amount of resveratrol loaded into the nanoparticles was quantified by HPLC-UV following an analytical method previously described [23] with minor modifications. Analysis were carried out in an Agilent model 1100 series LC coupled to a diode-array detector set at 306 nm. Data were analysed using Chemstation G2171 v. B.01.03 software (Agilent, USA). The chromatographic system was equipped with a reverse C18 Alltima column (150 mm x 2.1 mm, particle size 5 µm; Altech, USA) and a Gemini

C18 support AJO-7596 precolumn. The mobile phase, pumped at 0.25 mL/min was a mixture of water/methanol/acetic acid in a gradient condition. The column was heated at 40 °C and the injection volume was 10 µL. Under these conditions, the retention time for resveratrol was 22.8±0.5 min. Calibration curves in ethanol 75% were designed over the range of 1-100 µg/mL ($R^2 \geq 0.999$). Under these experimental conditions, the limit of quantitation was calculated to be 200 ng/mL.

For analysis, 10 mg nanoparticles were dispersed in 1 mL of water and centrifuged at 30,500 g for 20 min. The amount of encapsulated resveratrol was calculated by dissolution of the pellets with 1 mL of ethanol 75%. Each sample was assayed in triplicate and the results were expressed as the amount of resveratrol (µg) per mg of nanoparticles.

The encapsulation efficiency (E.E) was calculated as follows:

$$E.E.(\%) = \frac{Rsv\ p}{Rsv_t} \times 100 \quad \text{[Eq. 1]}$$

where Rsv-t is the total amount of resveratrol in the formulations and, Rsv-p, the amount of resveratrol quantified in the pellet.

In vitro release study

Release experiments were conducted under sink conditions at 37°C using simulated gastric (pH 1.2; SGF) and intestinal (pH 6.8; SIF) fluids [22], containing 0.5% Tween 20 as surfactant to increase the resveratrol aqueous solubility. The studies were performed under agitation in a slide-A-Lyzer® Dialysis cassete 10000 MWCO (Thermo scientific, Rockford, IL, USA). For this purpose, the cassette was filled with 3 mg of resveratrol nanoparticles previously dispersed in 5 mL water and, then, introduced in a vessel containing 500 mL of SGF (pH 1.2; 37ºC) under magnetic stirring. After 2 h in SGF, the cassette was introduced in another vessel containing 500 mL of thermostat-zed SIF (pH 6.8; 37ºC, under agitation). At different time points, samples were collected and filtered through 0.45 µm size-pore filters (Thermo scientific, Rockford,

USA) before quantification by HPLC. Calibration curves of resveratrol in SGF and SIF (0.05-6 µg/mL; R2 ≥ 0.999 in both cases) were performed.

In order to ascertain the resveratrol release mechanism the obtained data were fitted to the Korsmeyer-Peppas and the Peppas-Sahlin models. The Korsmeyer–Peppas model is a simple semi-empirical approach which exponentially relates drug release with the elapsed time as expressed in the following equation [24]:

$$\frac{M_t}{M_\infty} = \mathrm{K}_{KP} \cdot \mathrm{t}^{\mathrm{n}} \quad \text{[Eq. 2]}$$

where M_t/M_∞ is the drug release fraction at time t, K_{KP} is a constant incorporating the structural and geometric characteristics of the matrix and n is the release exponent indicative of the drug release mechanism [25]. Values close to 0.5 indicate a Case I (Fickian) diffusion mechanism and values between 0.5 and 0.89 indicate anomalous (non-Fickian) diffusion. Values of n between 0.89 and 1 indicate Case II transport, erosion of the matrix.

The contribution of Fickian and non-Fickian release was also evaluated by using the Peppas–Sahling model equation [26]:

$$\frac{M_t}{M_\infty} = \mathrm{K}_{\mathrm{D}} \cdot \mathrm{t}^{1/2} + \mathrm{K}_{\mathrm{E}} \cdot \mathrm{t} \quad \text{[Eq. 3]}$$

where the first term of the right-hand side is the Fickian contribution (K_D is the diffusional constant) and the second term is the Case II erosional contribution (K_E is the erosional constant). K_D and K_E values were used to calculate the contribution percentage of diffusion (D) and erosion (E) as follows [26]:

$$D = \frac{1}{1+\frac{K_E}{K_D}t^{0.5}} \quad \text{[Eq 4]}$$

$$\frac{E}{D} = \frac{K_E}{K_D}t^{0.5} \quad \text{[Eq 5]}$$

Only one portion of the release profile (Mt/M∞ ≤ 0.6) was used to fit the experimental data to the previous equation.

In vivo pharmacokinetic studies in Wistar rats

Pharmacokinetic studies

Pharmacokinetic studies were performed in male Wistar rats (200-250 g) obtained from Harlan (Barcelona, Spain). Studies were approved by the Ethical Committee for Animal Experimentation of the University of Navarra (protocol number 028-11) in accordance with the European legislation on animal experiments.

Prior to the oral administration of the formulations, animals were fasted overnight to avoid interference with the absorption, allowing free access to water. For the pharmacokinetic study, rats were randomly divided into 4 groups of 6 animals each. The three experimental groups were: (i) resveratrol water suspension (Rsv-susp), (ii) resveratrol solution in a PEG400:water mixture (Rsv-sol) and (iii) resveratrol-loaded zein nanoparticles (Rsv-NP-Z). As control, a group of animals was treated intravenously with the PEG400:water (1:1 by vol.) solution of resveratrol. Each animal received the equivalent amount of resveratrol to a dose of 15 mg/kg body weight either by oral gavage or intravenously via tail vein.

Blood samples were collected at set times after administration (0, 10 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, 24 h and 48 h) in specific plasma tubes (Microvette® 500K3E, SARSTEDT, Germany). Samples were immediately centrifuged at 9,400 g for 10 min and plasma aliquots were kept frozen at -80 ºC until HPLC analysis of both resveratrol and resveratrol-3-O-D-glucuronide.

Determination of resveratrol and resveratrol-3-O-D-glucuronide plasma concentration by HPLC

The amount of resveratrol was determined by HPLC-UV following an analytical method previously reported with minor modifications [27]. Analysis were carried out in an Agilent model 1100 series LC and diode-array detector set at 306 nm. Data were analysed in a Chemstation G2171 program (B.01.03). The chromatographic system was equipped with a reversed-phase C18 Kromasil column (250 mm x 2.1 mm; particle size 5 µm) and a Gemini C18 support AJO-7596 precolumn. The mobile phase, pumped at 0.5

mL/min, was a mixture of water, methanol and acetic acid (50:45:5 by vol.) under isocratic conditions. The column was thermostatized at 30°C and the injection volume was 30 µL. Under these conditions, the retention times for resveratrol-3-O-D-glucuronide and resveratrol were 6.2 ± 0.5 min and 12.6 ± 0.5 min, respectively.

For analysis, a 100 µL aliquot of plasma was mixed with 50 µL HCl 0.1 N and 500 µL acetonitrile (for protein precipitation) followed by vigorous shaking. Then, samples were centrifuged at 4000 rpm for 10 min and the obtained supernatants were evaporated under vacuum in a Speed Vac® system (Holbrook, NY) at 25°C for 30 min. Finally, 100 µL of a mixture of acetonitrile and water (1:1 by vol.) was added and vigorously stirred in a vortex for 10 min. Then, and prior to the injection, samples were filtered through 0.45 µm filter (Thermo scientific, Rockford, IL, USA).

For quantification, calibration curves were prepared over the range 2 to 70 µg/mL for the metabolite and 50 to 3,000 ng/mL for resveratrol ($R^2 \geq 0.99$). All the calibration standards were obtained by adding either resveratrol or resveratrol-3-O-D-glucuronide in acetonitrile (500 µL) to 100 µL plasma from non-treated animals. Then, the polyphenol or its metabolite was extracted using the same protocol described above.

Under these experimental conditions, the limit of quantification was calculated to be 70 ng/mL, for resveratrol, and 4 µg/mL for the metabolite. Linearity, accuracy and precision values during the same day (intra-day assay) at low, medium and high concentrations of both resveratrol and the metabolite were always within the acceptable limits (relative error and coefficient of variation less than 15%).

Pharmacokinetic data analysis

Resveratrol plasma concentration was plotted against time, and pharmacokinetic analysis was performed using a non-compartmental model with the WinNonlin 5.2 software (Pharsight Corporation, USA). The following parameters were estimated: maximal serum concentration (C_{max}), time in which C_{max} is reached (T_{max}), area under the concentration-time curve from time 0 to the last sampling-point (48 h) (AUC), mean residence time (MRT), clearance (Cl), volume of distribution (V) and half-life in the

293 terminal phase ($t_{1/2}$). Furthermore, the relative bioavailability (Fr %) of resveratrol was
294 estimated by the following equation:

295 $$Fr\ (\%) = \frac{AUC\ oral}{AUC\ iv} \times 100 \quad \text{(Eq. 6)}$$

296 where $AUC_{i.v.}$ and AUC_{oral} are the areas under the curve for the iv and oral
297 administrations, respectively.

298 ***In vitro/In vivo* correlation (INVIC)**

299 The eventual correlation between *in vitro* and *in vivo* results was conducted by plotting
300 a point-to-point between the amount of resveratrol released from nanoparticles *vs* the
301 fraction of resveratrol absorbed (FRA) calculated from the mean plasma concentration-
302 time inputs using the Wagner-Nelson equation [28]:

303 $$FRA = \frac{C_t + k \times AUC_{0-t}}{k \times AUC_{0-\infty}} \quad \text{(Eq. 7)}$$

304 where Ct is the plasma concentration of resveratrol at a time t, k is the elimination rate
305 constant of the polyphenol, AUC 0-t is the area under the resveratrol concentration *vs*.
306 time curve from 0 to time t, and AUC 0-∞ is the area under the curve from 0 to infinity.
307 Linear regression analysis was applied to the *in vitro–in vivo* correlation plot and
308 coefficient of determination (R^2) was calculated.

309 **Anti-inflammatory efficacy study**

310 **Animal model**

311 Four weeks-old (20-22 g) C57BL/6J female mice were purchased from Harlan
312 (Barcelona, Spain) and housed in standard animal facilities (6 animals per cage with
313 free access to food and drinking water). Housing conditions were maintained by
314 controlled temperature and humidity and with 12 h on/off light cycles. Animals were
315 allowed to acclimate for one week before the experiment.
316 *In vivo* anti-inflammatory studies were evaluated in an endotoxic shock model set up by
317 intraperitoneal (ip) administration of LPS at a dose of 40 µg per mouse 29. Before
318 administration, LPS was dissolved in PBS and vortexed during 30 min to complete
319 homogenization.

On day 1, mice were randomly distributed into four groups. The first group of animals received an oral dose of 15 mg/kg resveratrol daily as oral solution (Rsv-sol) during 7 days. The second group of animals received the same posology of polyphenol (15 mg/kg resveratrol daily; 7 days) but formulated in zein nanoparticles (Rsv-NP-Z).. As controls, a group of animals received LPS treatment (positive control group) and another one received neither LPS nor resveratrol (negative control group).

Twenty-four hours after the last dose of resveratrol (day 8) animals were challenged with 40 µg LPS by ip route. Throughout the study, rectal temperature of mice was measured until 24 h after challenge. Similarly, animals were observed for any clinical signs or symptoms of toxicity daily and after the challenge. The severity of symptoms was scored as follows:: i) (-) absent; ii) (+) weak; iii) (++) moderate; and iv) (+++) strong. Depending on the activity of animals, their mobility was classified as very low, low or normal.

In addition, 90 min after challenge, blood samples were collected from the retro-orbital cavity in EDTA-K vials (Microvette® 500K3E, SARSTEDT, Germany), centrifuged at 8,000 g for 10 min for sera collection and stored at -20 °C until use.

Measurement of plasma TNF-α

The concentration of circulating TNF-α in the serum was determined by an enzyme-linked immunosorbent assay kit (Quantikine® ELISA Mouse TNF-α, MTA00B, R&D Systems, Minneapolis, USA) according to manufacturer's instructions.

Statistical analysis

Data are expressed as the mean ± standard deviation (S.D.) of at least three experiments. The non-parametric Kruskall-Wallis followed by Mann-Whitney U-test was used to investigate statistical differences. In all cases, $p < 0.05$ was considered to be statistically significant. All data processing was performed using Graph Pad® Prism statistical software.

Results

348 **Preparation and characterization of nanoparticles**

349 **Table 1** shows the physico-chemical characteristics of the nanoparticles used in this
350 study. Overall, the mean diameter of empty nanoparticles was smaller than those
351 loaded with resveratrol. When resveratrol was encapsulated, zein nanoparticles
352 displayed a mean size of about 310 nm, whereas, the polydispersity index was found to
353 be lower than 0.2, indicating homogeneous nanoparticle formulations. Furthermore, the
354 zeta potential of nanoparticles was negative (- 51 mV); however, when resveratrol was
355 encapsulated the resulting nanoparticles were slightly more negative than for empty
356 ones (**Table 1**). Additionally, the resveratrol loading was calculated to be about 80
357 µg/mg nanoparticles, with an encapsulation efficiency close to 82%.

358 **Figure 1** shows the morphology and shape of resveratrol-loaded nanoparticles. In all
359 cases, nanoparticles consisted of homogeneous populations of spherical particles with
360 a smooth surface. In addition, the size of nanoparticles as observed by SEM was in line
361 with the values determined by photon correlation spectroscopy (**Table 1**).

362 **In vitro release profile**

363 **Figure 2A** represents the release profile of resveratrol from nanoparticles expressed
364 as cumulative percentage of drug released *versus* time. In all cases, the release of
365 resveratrol from zein-based nanoparticles was found to be independent of the pH
366 conditions. During the first 2 h, under SGF conditions (pH 1.2), about 20% of the
367 loaded resveratrol was released from zein nanoparticles. Then, 6 hours later (during
368 incubation in SIF conditions) the amount released was close to 60% of the total content
369 of resveratrol. After 48 h, all the loaded resveratrol was released from nanoparticles.

370 The release profile of resveratrol from NPs was fitted to different mathematical release
371 models. Using the Korsmeyer-Peppas equation, R^2 values were high (R^2>0.96) and the
372 exponent “n” value was 0.75±0.06. All of this suggests that the release of resveratrol
373 from nanoparticles would be a combination of Fickian diffusion and erosion of the
374 nanoparticle matrix. Under these circumstances, the Peppas-Sahlin model was applied
375 and the erosion (K_E) and diffusion (K_D) constants were calculated (K_D = 0.08±0.02 $h^{-1/2}$;

K_E: 0.04±0.01 h^{-1}). **Figure 2B** displays the contribution of both the diffusion and erosion mechanisms on the release of resveratrol from zein nanoparticles. The time at which both mechanisms (diffusion and erosion) contributed in a similar amount to the release of resveratrol was calculated to be 3.5 h.

In vivo pharmacokinetics

Figure 3A shows the plasma concentration-time profile of a resveratrol solution in PEG-400:water (1:1 by vol.) after the intravenous administration to rats of a single dose of 15 mg/kg. The data were adjusted to a non-compartmental model. The resveratrol plasma concentration decreased rapidly in a biphasic way during the first 8-h post administration. The peak plasma concentration (C_{max}) of resveratrol was around 15 µg/mL, whereas the AUC and half-life ($t_{1/2}$) were calculated to be 11.4 µg h/mL and 2.0 h, respectively. The resveratrol clearance and its volume of distribution were about 0.2 L/h and 0.6 L, respectively (**Table 2**).

Figure 3B shows the plasma concentration levels of resveratrol when administered orally as a single dose of 15 mg/kg to rats. Interestingly, when resveratrol was formulated as a suspension, no detectable levels of the polyphenol were quantified in plasma. On the other hand, when resveratrol was administered as solution (Rsv-sol), the polyphenol plasma levels displayed an initial maximum concentration (C_{max}) of around 0.2 µg/mL, 30 min after administration. Then, the plasma levels of resveratrol decreased rapidly and quantifiable levels were only detected during the first 4 h post-administration.

For resveratrol-loaded in zein nanoparticles (Rsv-NP-Z), the amount of the polyphenol in plasma increased during the first 4 h after administration until reaching a maximum. Then, the resveratrol plasma levels decreased slowly for the following 20 h. Forty-eight hours post-administration, the amount of resveratrol in plasma was very close to the quantitation limit of the analytical technique.

Table 2 summarizes the main pharmacokinetic parameters estimated with a non-compartmental analysis of the experimental data obtained after the administration of

404 the different formulations to rats. The resveratrol AUC values from zein nanoparticle
405 formulations were significantly higher ($p < 0.05$) than those observed for the polyphenol
406 solution. Similarly, the resveratrol MRT was thirteen-times higher when administered in
407 the form of zein nanoparticles than when solubilized in the PEG400:water oral mixture.
408 Finally, the relative oral bioavailability of resveratrol when incorporated in nanoparticles
409 was calculated to be 50 % using zein nanoparticles. This value was significantly higher
410 than the bioavailability obtained with the PEG400:water solution (2.6 %).
411 **Figure 4** shows the plasma concentration *versus* time profile of the resveratrol main
412 metabolite (resveratrol-O-3-glucuronide) after the single administration of the
413 polyphenol in the formulations tested. Interestingly, the profile of the plasma curves for
414 both resveratrol and its metabolite were similar; however, the metabolite levels were
415 always higher than for the polyphenol. When resveratrol was administered
416 intravenously, the metabolite concentration reached 41.9 µg/mL (C_{max}) and, then, the
417 metabolite levels decreased sharply. The AUC value was calculated to be 197 µg
418 h/mL.
419 For the solution of resveratrol orally administered, the C_{max} of the metabolite in plasma
420 was found to be 2-times lower (22.1 µg/mL) than when administered by the iv route. In
421 this case, the metabolite was only quantified in plasma during the first 8 h post-
422 administration. The AUC value was calculated to be 104 µg h/mL; around half the i.v.
423 solution one.
424 For nanoparticles, the metabolite was quantified during the first 24 hours after
425 administration. In addition, the metabolite AUC data was around 342 µg h/mL for Rsv-
426 NP-Z. This value was around three-times higher than with the resveratrol was
427 administered as oral solution or intravenously.
428 **In vitro-in vivo correlations**
429 **Figure 5** represents the relationship between the *in vitro* dissolution data (expressed
430 as the cumulative percentage of the polyphenol released) and the fraction of

resveratrol absorbed during the first 8 h post-administration. An acceptable linear regression was observed between both data (R^2 = 0.83 for Rsv-NP-Z).

Anti-inflammatory efficacy study

Figure 6A shows rectal temperature of mice for 24 h after ip administration of 40 µg LPS Before challenge, all the animals displayed a similar rectal temperature (data not shown). However, six hours after challenge, important differences were observed among groups. Thus positive control animals (without any resveratrol treatment) displayed a body temperature of about 4ºC below the basal normal levels. For animals treated with Rsv-sol the body temperature was 3ºC lower than before challenge. On the contrary, rectal temperature of animals treated with resveratrol loaded in zein nanoparticles, decreased only 0.5-1 °C. No variations were observed in the control negative group. Twenty-four hours after challenge animals treated with free resveratrol or encapsulated regained normal temperature.

Table 3 shows the overall endotoxic symptoms score including the number of animals displaying a temperature 2 ºC lower than the basal temperature, 6 h post-challenge. Positive control animals displayed a low mobility and signs of bristly hair and respiratory distress. On the contrary, animals treated with Rsv-NP-Z displayed an almost normal behaviour and an evident better symptomatology than those animals receiving resveratrol as oral solution, which appeared to be immobile or with a high difficulty to coordinate any simple movement.

Figure 6B shows the serum levels of TNF-α measured by ELISA before and 90 min after LPS challenge. Negligible levels of TNF-α were observed before LPS administration. The oral administration of Rsv-NP-Z induced a decrease in the levels of TNF-α with respect to mice pre-treated with resveratrol solution and the positive control group; however, these differences were not statistically significant. Significant differences ($p<0.01$) were observed between control negative and the rest of groups.

Discussion

459 In the past zein was proposed as material for the preparation of nanoparticles due to its
460 hydrophobic character, degradability, adherence properties and versatile processability
461 [19,20]. However, as zein possesses abundant non-polar amino acids, the dispersability of
462 the resulting nanoparticles in an aqueous media (and, therefore, their potential
463 applications) is a challenge [30]. Recently, the use of citrate and phosphate salts was
464 proposed to minimize this problem [31]. In our case, lysine was added during the
465 preparative process of nanoparticles. In this way, the resulting dry powder of zein
466 nanoparticles was easily redispersed, yielding a homogeneous fine suspension (**Table**
467 **1**) after the addition of water and simple hand agitation.
468 Resveratrol-loaded zein nanoparticles (Rsv-NP-Z) displayed a mean size close to 300
469 nm and negative zeta potential. The resveratrol loading was of 80 µg/mg nanoparticles
470 with an encapsulation efficiency of 80%. This payload is in line with values previously
471 reported by using solid lipid nanoparticles [17], PLGA nanoparticles [18], or nanoemulsions
472 [32]. The release of resveratrol from zein nanoparticles was found to be pH-independent.
473 In fact, this phenomenon would be a combination of both Fickian diffusion and erosion
474 of the nanoparticle matrix (Peppas-Sahlin model). During the first hours of the release
475 process, resveratrol molecules would mainly diffuse from the nanoparticles to the
476 aqueous medium by Fickian diffusion. Later (3.5 h), the release of resveratrol would be
477 mainly due to an erosion and/or relaxation process of the nanoparticle matrix.
478 Interestingly, as a consequence of both phenomena, the amount of resveratrol
479 released (at least during the first 8 h) is constant and approaches to a zero order
480 kinetic.
481 Pharmacokinetic studies were carried out at a single dose of 15 mg/kg, comparable to
482 those used in previous studies [33,34]. The oral administration of a single dose of
483 resveratrol as an aqueous suspension (Rsv-susp) to rats did not produce quantifiable
484 levels of the polyphenol in plasma (**Figure 3B**). For the solution formulation, in a
485 PEG400:water mixture (Rsv-sol), the plasma levels of the polyphenol were higher than
486 for the suspension but they rapidly decreased and 6 h-post administration only traces

487 of resveratrol in plasma were detected. These findings are directly related with the
488 extensive metabolism of resveratrol [35]. In fact, when administered orally, resveratrol
489 (due to its lipophilic character) can rapidly enter into the enterocyte by passive diffusion
490 [36]; although, it is highly metabolized to glucuronide and sulphate derivatives, which may
491 be secreted back to the intestinal lumen through multidrug resistance protein 2 (MRP2)
492 and BCRP [37,38]. This extensive biotransformation of resveratrol decreases circulation
493 levels of free resveratrol and facilitates its excretion (in the form of conjugates) by the
494 kidneys via urine [14]. Controversy remains about the physiological activity of metabolites
495 or if they can act as resveratrol prodrugs. There are evidences that, at sufficient
496 concentrations, resveratrol metabolites have biological activity in various tissues [36].
497 Nevertheless, there are also evidences that these compounds have no effects in some
498 tissues [39].
499 However, when resveratrol was administered after its encapsulation in zein
500 nanoparticles, sustained and prolonged plasma levels of the polyphenol were observed
501 for at least 24 h (**Figure 3B**) and its relative oral bioavailability was about 50% (**Table**
502 **3**), which is about 18-fold higher than the value observed for Rsv-sol (about 2.6%). This
503 increased capability to promote the absorption and bioavailability of resveratrol by
504 using zein nanoparticles would be related with its high hydrophobic character, that
505 would offer a higher stability in vivo, and to the capability of this corn protein to develop
506 mucoadhesive interactions within the gut mucus layer [40]. Thus, this characteristic would
507 provide a longer residence in close contact with the intestinal epithelium and facilitating
508 the establishment of a concentration gradient from the nanoparticulate matrix untill the
509 absorptive membrane. Interestingly, the fraction of resveratrol absorbed from zein
510 nanoparticles correlated well with the percentage of the polyphenol released *in vitro*
511 (see **Figure 5**).
512 In previous studies, it has been reported that the oral bioavailability of resveratrol is
513 almost zero [34,41]. In order to improve its absorption different strategies have been
514 proposed such as the use of oral absorption enhancers (e.g. Tween 80, cyclodextrins)

515 or the employment of resveratrol derivatives. In this way, Kapetanovic and co-workers
516 have reported an oral bioavailability of resveratrol (formulated as aqueous solution
517 containing methylcellulose and Tween 80) close to 30% after the administration of a
518 single dose of 50 mg/kg in rats. In the same work, the administration of the same
519 resveratrol formulation at a dose of 150 mg/kg produced an oral bioavailability of 19%
520 [42]. In another work, resveratrol trimethyl-ether administered orally in a solution
521 formulated with randomly methylated-β-cyclodextrin (15 mg/kg) yielded a bioavailability
522 of about 47%. More recently, the use of nanocarriers has also been proposed. Thus, in
523 mice and using a dose of 50 mg/kg, the oral bioavailability of resveratrol when loaded
524 in either Eudragit or chitosan/lecithin nanoparticles was calculated to be 39 and 61%,
525 respectively [43]. For solid lipid nanoparticles, the oral bioavailability of the polyphenol
526 was found to be 8-fold higher than for a conventional solution of resveratrol [44]. In our
527 case, the resveratrol bioavailability was 18-fold higher when loaded in zein
528 nanoparticles than when dissolved in the PEG400:water solution. Furthermore, zein
529 nanoparticles offering sustained and prolonged levels of resveratrol in plasma provided
530 a supplementary advantage when compared with other strategies.
531 Regarding the presence of the main metabolite (resveratrol-O-3-glucuronide [13]) in the
532 plasma of animals, the levels of this compound (measured as AUC) were higher when
533 resveratrol was administered encapsulated in zein nanoparticles than when
534 administered in the conventional solution both by iv route (about 1.7 times) or orally
535 (around 3.3 times). This fact would be related with the slow release of the polyphenol
536 from the nanoparticles (where protected from degradation) and a prolonged residence
537 of nanoparticles in the gut mucosa due to their mucoadhesive properties. In other
538 words, by using nanoparticles, more resveratrol and during a longer period would reach
539 the circulation, counterbalancing the natural rapid metabolism of the drug.
540 Finally, we studied the anti-inflammatory activity of resveratrol when loaded in zein
541 nanoparticles. Several *in vitro* and *in vivo* studies suggest that resveratrol inhibits the
542 inflammatory response mediated by microbial stimuli [45]; by inhibiting the transcription

543 factor NF-κB [10,11]. Therefore, we tested here the protective effect of encapsulated
544 resveratrol against the inoculation of LPS in mice. LPS is present exclusively on the
545 outer membrane of Gram negative bacteria, and consequently, it is one of the most
546 strong alarm signals for the innate immune system, inducing in animals a
547 pathophysiologic syndrome known as endotoxic shock. This syndrome is similar to
548 sepsis shock syndrome that progress on multiple organ failure [29,46], showing
549 piloerection, hypothermia, shivering, tachycardia and lethargy. These symptoms are
550 related with large amounts of released inflammatory mediators, such as TNF-α, NO
551 and prostaglandin E2 (PGE2), where TNF-α play a central role as being the first one to
552 be released [10]. In our experimental conditions, untreated mice challenged with LPS
553 (positive control) displayed the highest decrease in rectal temperature and the highest
554 TNF-α serum level. In contrast, Rsv-NP-Z administered daily during 7 days, were able
555 to diminish endotoxic symptoms like hypothermia or piloerection and increase the
556 movement of mice compared to those treated with resveratrol solution on daily basics
557 (**Figure 6**, **Table 3**). Moreover, for animals treated with Rsv-NP-Z, TNF-α levels were
558 lower than for controls; although the high variability of values abolished the statistical
559 significance. These results appear to indicate that the presence of sustained high
560 levels of resveratrol in plasma could be efficient to reduce the inflammatory mediators
561 in endotoxic shock induced by LPS.
562 In summary, zein nanoparticles appear to be interesting carriers for the oral delivery of
563 resveratrol. The polyphenol is released from this carrier by a combination of both
564 diffusion and erosion of the nanoparticle matrix, providing higher and more prolonged
565 plasma levels of resveratrol up to 48 h. Consequently, these nanocarriers significantly
566 increased the oral bioavailability of resveratrol reaching a value close to 50%. The oral
567 administration of these nanoparticles during one week to mice challenged with LPS
568 protected them from the inflammatory symptoms and mediators of the endotoxic shock.
569 Future studies should be performed to ascertain how this treatment modulates TNF-α

570 production in order to explore the potential use of Rsv-NP-Z as anti-inflammatory
571 treatment.
572
573 ## Acknowledgements
574 This work was supported by the Regional Government of Navarra (Alimentos
575 funcionales, Euroinnova call) and the Spanish Ministry of Science and Innovation and
576 Gobierno de Navarra (ADICAP; ref. IPT-2011-1717-900000). Rebeca Penalva
577 acknowledges the “Asociación de Amigos Universidad de Navarra” for the financial
578 support.
579

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702 induced expression of HMGB1 and TLR4 in RAW264.7 cells. *Cell Physiol Biochem*. **2014**, *33*,
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704

705 **Figure captions**

706 **Figure 1**. Scanning electron microscopy (SEM) microphotograph of resveratrol-loaded
707 zein nanoparticles. Bar indicates the resolution (1 μm). The white box delimits a
708 magnified area.

709 **Figure 2**. Resveratrol release from zein-based nanoparticles (Rsv-NP-Z). A)
710 Resveratrol release profile when incubated in simulated gastric (SGF, pH 1.2; 0-2 h)
711 and simulated intestinal fluids (SIF, pH 6.8; 2-48 h) under sink conditions. Data
712 represented as mean ± SD (n=3). B) Fraction contribution of the Fickian diffusion (●)
713 and the erosion/relaxation (○) mechanisms to resveratrol release from zein
714 nanoparticles (Rsv-NP-Z).

715 **Figure 3**. Resveratrol plasma concentration vs time after a single administration of the
716 polyphenol at a dose of 15 mg/kg. A) Intravenous administration of the resveratrol
717 solution in the PEG400:water mixture. B) Oral administration of the following
718 resveratrol formulations: i) resveratrol suspension (Rsv-susp, ▲), ii) resveratrol solution
719 (Rsv-Sol, ♦) and iii) resveratrol-loaded zein nanoparticles (Rsv-NP-Z, ■). Data
720 expressed as mean ± SD (n=6).

721 **Figure 4**. Resveratrol-O-3-glucuronide concentration vs time after a single
722 administration (intravenous or oral) of the different formulations at dose of 15 mg/kg. i)
723 Resveratrol intravenous (Rsv-IV, ◊) ii) Oral resveratrol solution (Rsv-Sol, ▲), and iii)
724 Oral resveratrol loaded in zein nanoparticles (Rsv-NP-Z, ■). Data expressed as mean ±
725 SD, n= 6.

726 **Figure 5**. Relationship between fractions dissolved in vitro vs. fraction absorbed in vivo
727 of Resveratrol loaded into zein nanoparticles (Rsv-NP-Z). FRD (fraction of resveratrol
728 dissolved), FRA (fraction of resveratrol absorbed).

Figure 6: Anti-inflammatory activity of resveratrol. A) Comparative of decreased rectal temperature of mouse after ip administration of LPS (40 µg) on time. B) TNF-α serum levels before and 1.5 h post LPS (40 µg) administration. Mice were pre-treated orally daily for 7 days with resveratrol loaded in zein nanoparticles (Rsv-NP-Z) or resveratrol solubilized in PEG400-H2O (Rsv-sol) (1:1 by vol.). No pre-treated with resveratrol (control +) and negative controls (no pretreated with resveratrol and no treated with LPS) were also included. Results expressed as mean ± SD (n=6).***p<0.01 Kruskal Wallis test.

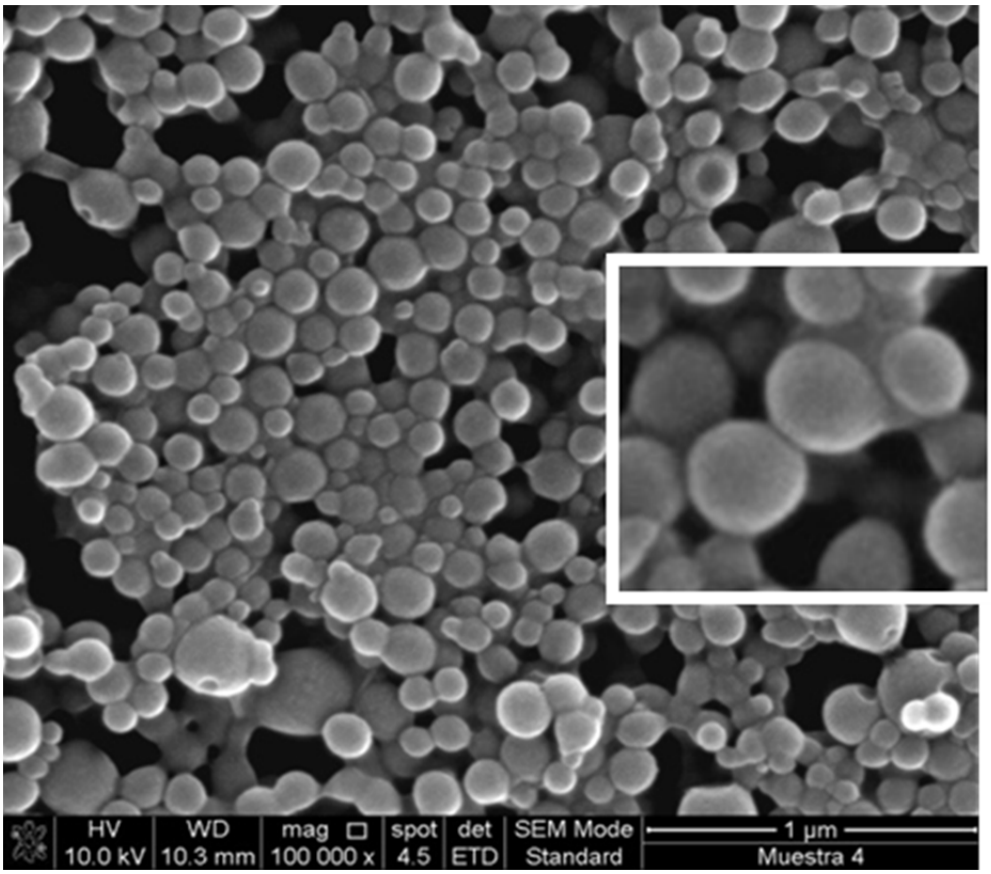

HV
10.0 kV
WD
10.3 mm
mag
100 000 x
spot
4.5
det
ETD
SEM Mode
Standard
1 µm
Muestra 4

Figure 1.

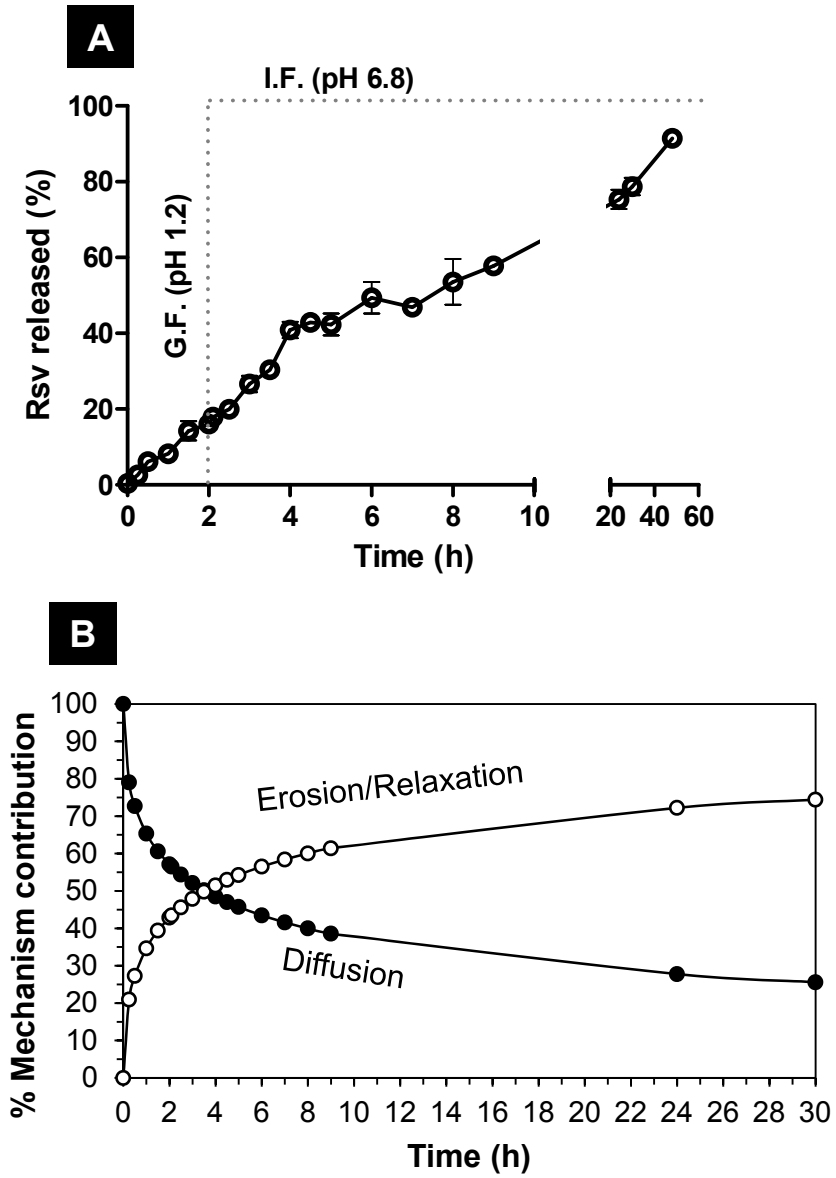

A
I.F. (pH 6.8)
G.F. (pH 1.2)
Rsv released (%)
Time (h)
B
% Mechanism contribution
Erosion/Relaxation
Diffusion
Time (h)

Figure 2.

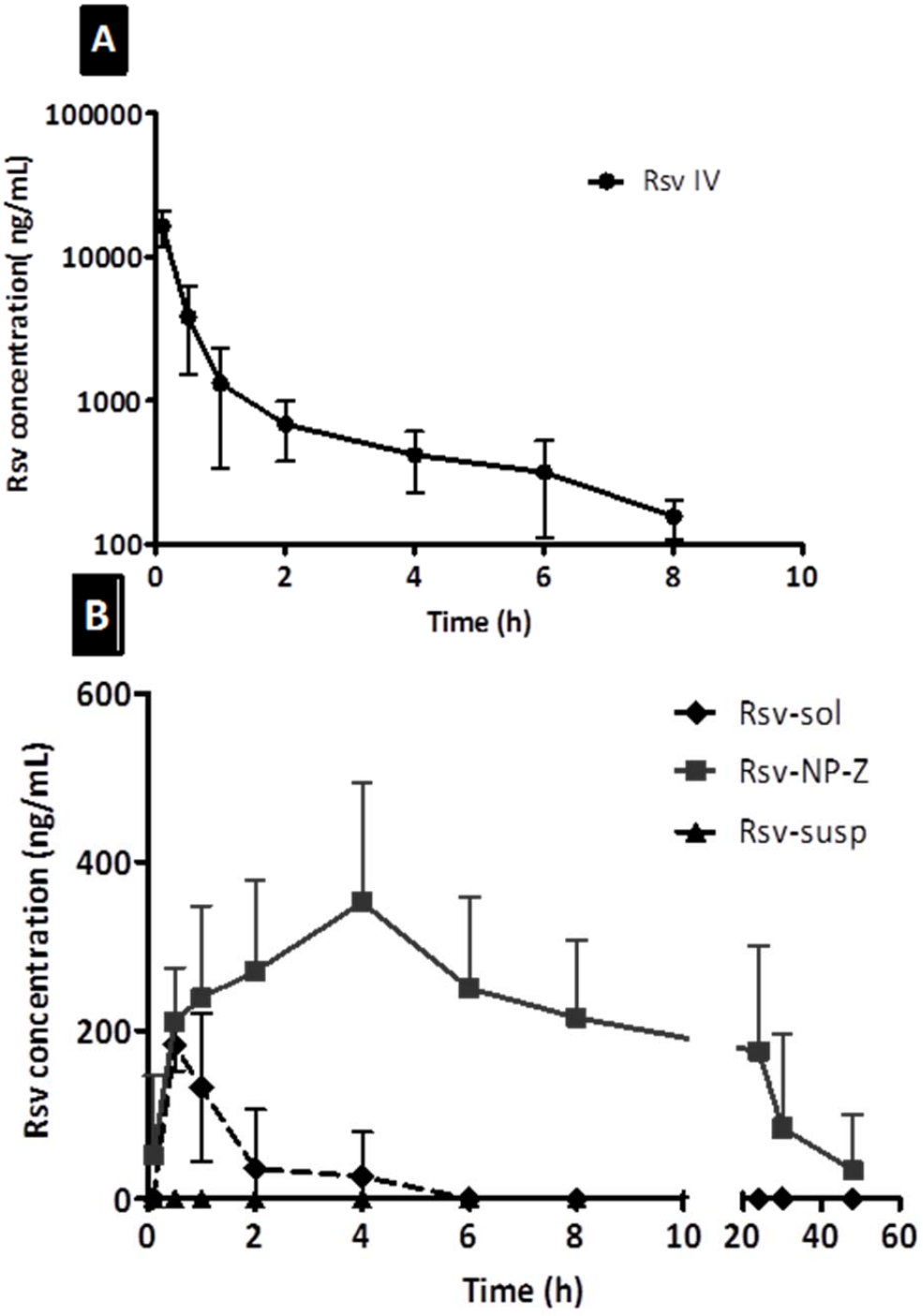

A
100000
10000
1000
100
Rsv concentration(ng/mL)
Rsv IV
0
2
4
6
8
10
Time (h)
B
600
400
200
0
Rsv concentration (ng/mL)
Rsv-sol
Rsv-NP-Z
Rsv-susp
0
2
4
6
8
10
20
40
60
Time (h)

Figure 3.

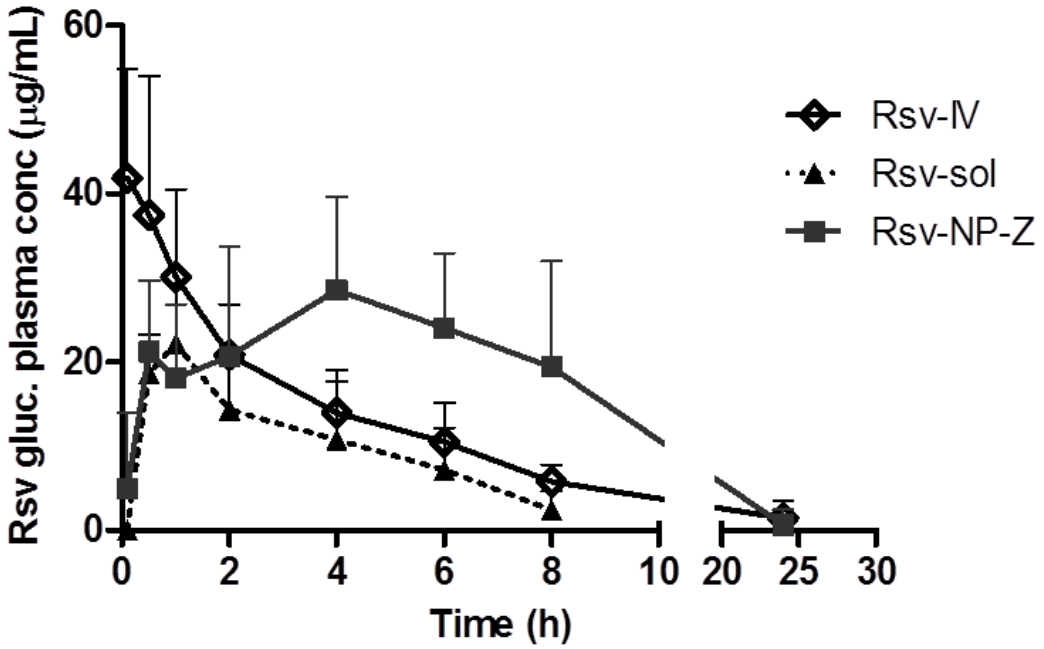

Rsv gluc. plasma conc (μg/mL)
60
40
20
0
0
2
4
6
8
10
20
25
30
Time (h)
Rsv-IV
Rsv-sol
Rsv-NP-Z

Figure 4.

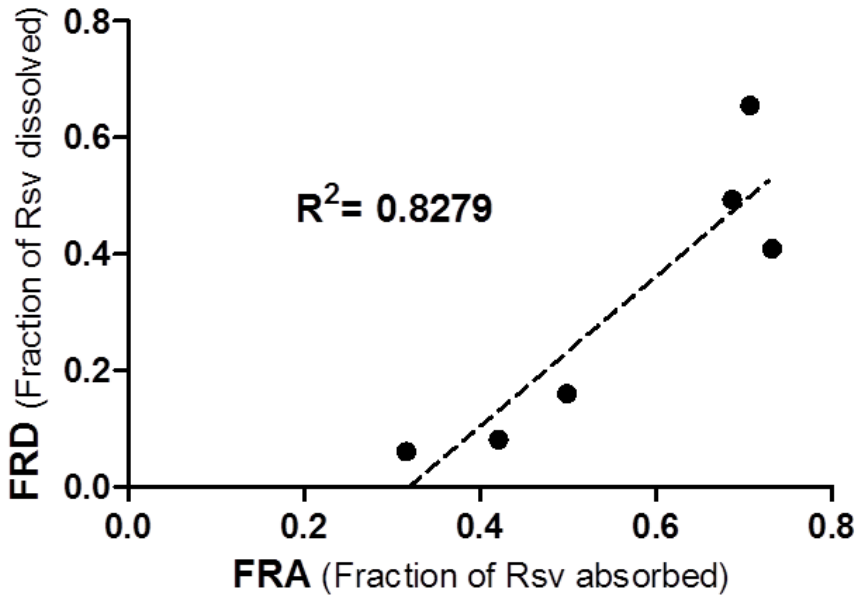

FRD (Fraction of Rsv dissolved)
0.8
0.6
0.4
0.2
0.0
R2= 0.8279
0.0
0.2
0.4
0.6
0.8
FRA (Fraction of Rsv absorbed)

Figure 5.

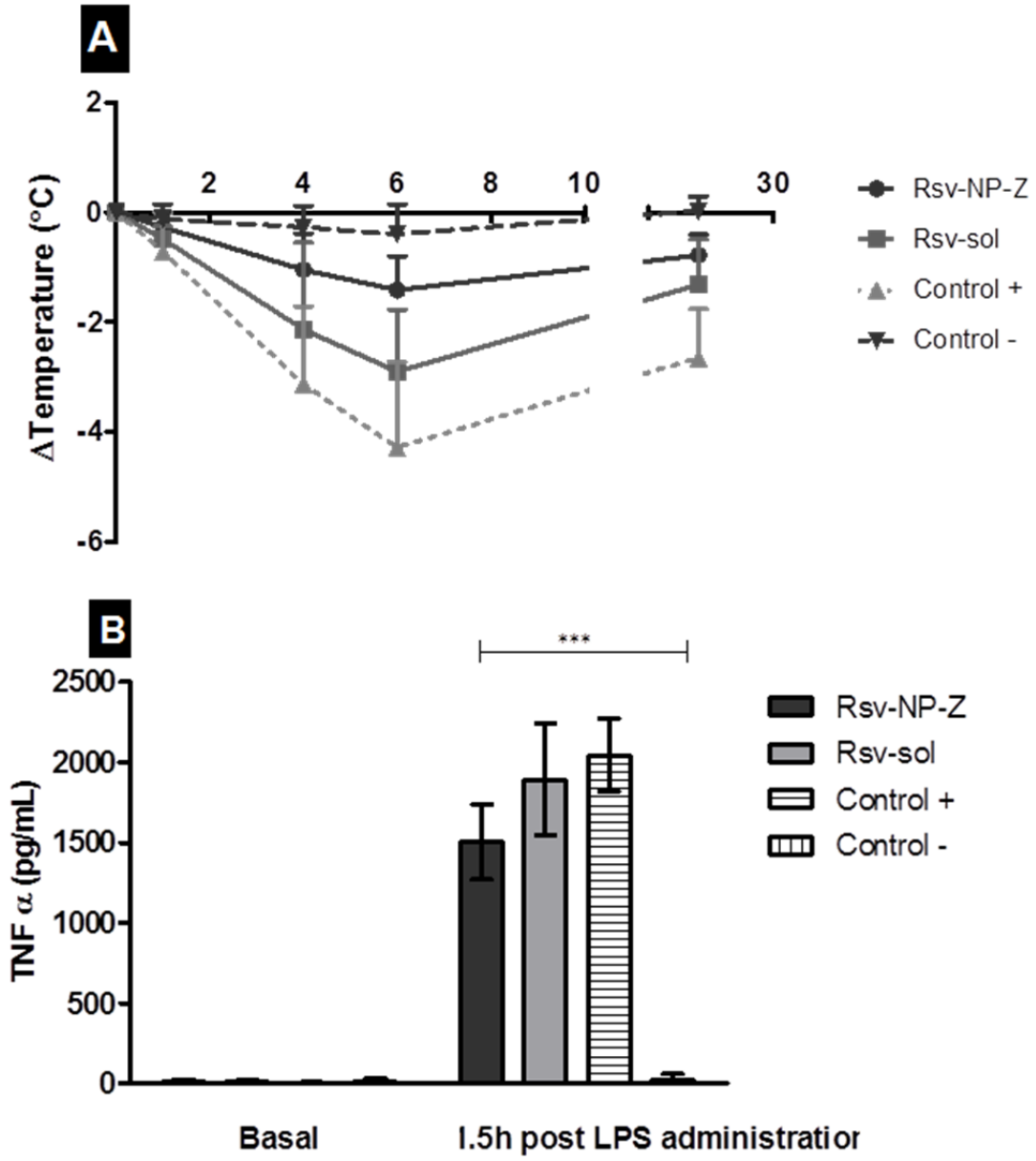

A
2
0
-2
-4
-6
ΔTemperature (°C)
2
4
6
8
10
30
Rsv-NP-Z
Rsv-sol
Control +
Control -
B
***
2500
2000
1500
1000
500
0
TNF α (pg/mL)
Rsv-NP-Z
Rsv-sol
Control +
Control -
Basal
l.5h post LPS administratior

Figure 6.

Tables

Table 1. Physico-chemical characteristics of empty and resveratrol-loaded nanoparticles. NP-Z: empty zein nanoparticles; Rsv-NP-Z: resveratrol-loaded zein nanoparticles. PDI: polydispersity index. Data expressed as mean ± SD, n=6.

	Size (nm)[a]	PDI	Zeta potential (mV)	Rsv loading (µg/mg NP)[b]	E.E. (%)[c]
NP-Z	264 ± 2	0.07 ± 0.01	-46 ± 2		
Rsv-NP-Z	307 ± 3	0.10 ± 0.01	-51 ± 0	80 ± 3	82 ± 4

[a] Determination of volume mean diameter by photon correlation spectroscopy

[b] Determination of resveratrol content by HPLC-UV

[c] Encapsulation efficiency (%)

Table 2. Pharmacokinetic parameters of resveratrol obtained after the administration of the different formulations tested at a dose of 15 mg/kg to Wistar male rats. i) Resveratrol intravenous (Rsv-iv) ii) Rsv solution (Rsv-sol), iii) Resveratrol suspension (Rsv-susp) and iv) Resveratrol loaded in zein nanoparticles (Rsv-NP-Z). Data expressed as mean ± SD. (n=6)

	Route	C_{max} (µg/mL)	T_{max} (h)	AUC (µg h/mL)	T ½ (h)	Cl (mL/h)	Vd (mL)	MRT (h)	Fr (%)
Rsv iv.	iv	15.2± 5.18	0.1±0.0	10.4 ± 3.80	2.0±0.5	199 ± 89.8	569 ± 221	2.4±1.0	100
Rsv-sol	oral	0.20 ± 0.02*	0.6±0.2	0.28 ± 0.13*	0.3±0.2	387 ± 225	112 ±104	1.3±0.8	2.6
Rsv-susp	oral	ND	ND	ND	ND	ND	ND	ND	ND
Rsv-NP-Z	oral	0.39 ± 0.11*†	4.9 ± 3.1	5.17 ± 2.61†	5.5 ± 1.7	125 ± 41	909 ± 184	17.1 ± 7.1*†	50.0

C_{max}: peak plasma concentration; T_{max}: time to reach plasma concentration; AUC: Area under the curve; t ½: half life of the terminal phase; Cl: Clearance; MRT: mean residence time Fr: relative oral bioavailability

† Significant differences *vs* Rsv-Sol ($p<0.05$) Mann-Whitney-U

* Significant differences *vs* Rsv-i.v. ($p<0.01$) Mann-Whitney-U

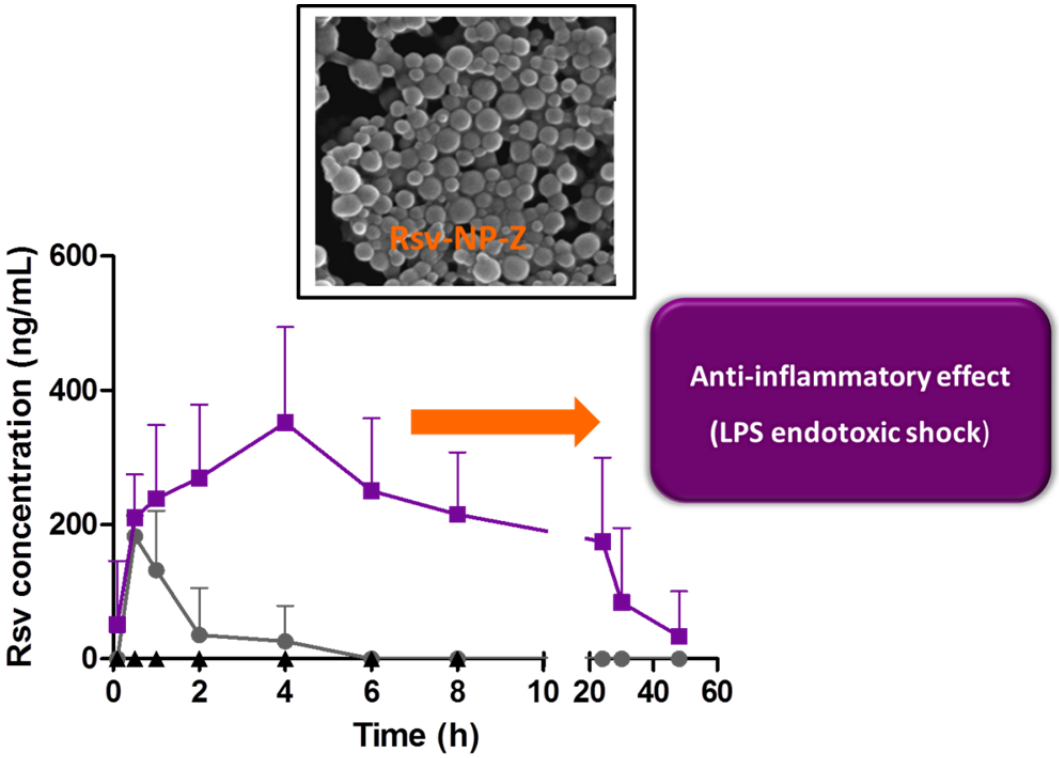
Table 3. Endotoxic symptoms in the resveratrol treated *vs* no treated LPS-inoculated mice.

Treatment*	T^a decreased** >2°C	Piloerection	Mobility
Control -	0/6	-	Normal
Control +	6/6	+++	Very low
Rsv-Sol	4/6	++	Very Low
Rsv-NP-Z	1/6	+	Low

*Control -: No treated, no LPS; Control +: No treated but inoculated with LPS; Rsv-Sol: administration of resveratrol solution daily during 7 days, LPS; Rsv-NP-Z: administration of resveratrol-loaded zein nanoparticles daily during 7 days, LPS. (n=6).Severity of the symptoms: (-) None; (+) weak; (++) moderate; (+++) strong.

**, Decreased of temperature 6 h after LPS inoculation.

TOC Graphic


Rsv-NP-Z
Rsv concentration (ng/mL)
600
400
200
0
0
2
4
6
8
10
20
40
60
Time (h)
Anti-inflammatory effect
(LPS endotoxic shock)