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

Authors

Rebeca Penalva a, Irene Esparza a, Eneko Larraneta a, Carlos J. González-Navarro c, Carlos Gamazo b, Juan M. Irache a

Affiliation

a Department of Pharmacy and Pharmaceutical Technology, University of Navarra, 31008 - Pamplona, Spain.
b Department of Microbiology. University of Navarra, 31008 - Pamplona, Spain.
c Centre for Nutrition Research, University of Navarra, 31080 - Pamplona, Spain.

Corresponding author:
Prof. Juan M. Irache
Dep. Pharmacy and Pharmaceutical Technology
University of Navarra
C/ Irunlarrea, 1
31080 – Pamplona
Spain
Phone: +34948425600
Fax: +34948425619
E-mail: jmirache@unav.es

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
Cmax: maximal serum concentration
Tmax: 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
t1/2: 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. Resveratrol has been classified as a phytoalexin as it is synthesized in spermatophytes in response to injury, UV irradiation and fungal attack. 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.

Resveratrol offers pleiotropic health beneficial effects, including antioxidant and anti-aging effects, cardioprotective, anticancer, neuroprotective and HIV/AIDS activities.

In the last 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. Similarly, resveratrol was reported to reduce body weight and adiposity in obese recipients. These actions would involve the activation of sirtuin 1 (SIRT1) that inhibits inflammatory pathways in macrophages and modulates insulin sensitivity. Furthermore, different studies have shown that resveratrol is capable of inhibiting lipopolysaccharide (LPS) induced cytokine production. This effect, via modulation of NF-κB, would decrease the production and gene expression of IL-1 and TNF-α, important endogenous pyrogens.

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. This low bioavailability is due to the polyphenol biotransformation by UDP-glucuronosyltransferase and sulphotransferases that produces resveratrol-3′-glucuronide and the sulphate derivative, respectively. In rats, the main metabolite of resveratrol is the glucuronide conjugate, whereas, in humans, both the glucuronide and the sulphate derivatives have been described. These metabolites have a longer plasma half-life, however, their efficacy are unknown.
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-nanoemulsifying 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) \(\alpha\)-zein (75-85% of total protein) with two main MW of 21-25 kDa and 10kDa, ii) \(\beta\)-zein (10-15%) of a MW of 17-18 kDa, iii) \(\delta\)-zein, a minor fraction of 10kDa and vi) \(\gamma\)-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-
D-glucuronide (Rsv-O-glu) was from @rtMolecule (Poitiers, France). Ethanol, methanol, acetic acid and acetonitrile HPLC grade were obtained from Merck (Darmastadt, Germany). Lipopolysaccharide from Salmonella enterica serovar. Minnesota (LPS) was purchased from Sigma®, (St. Louis, USA). Deionised reagent water (18.2 MO resistivity) was prepared using a water purification system (Wasserlab, Spain). All reagents and chemicals used were of analytical grade.

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

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

Control formulations (NP-Z) were prepared as described above but in absence of resveratrol.

**Preparation of resveratrol conventional formulations**

Two different formulations of resveratrol were also prepared. The first one, a solution of 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.

**Resveratrol analysis**

The amount of resveratrol loaded into the nanoparticles was quantified by HPLC-UV following an analytical method previously described 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
triple and the results were expressed as the amount of resveratrol (µg) per mg of
nanoparticles.

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

$$E.E. (\%) = \frac{Rsv_{p}}{Rsv_{t}} \times 100 \quad [Eq. 1]$$

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, 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 cassette 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; 37°C) under magnetic stirring. After 2 h in
SGF, the cassette was introduced in another vessel containing 500 mL of thermostat-
ized SIF (pH 6.8; 37°C, 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:

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

where \(M_t / M_\infty\) 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:

\[
\frac{M_t}{M_\infty} = K_D \cdot t^{1/2} + K_E \cdot t \quad [\text{Eq. 3}]
\]

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:

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

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

Only one portion of the release profile (\(M_t / M_\infty \leq 0.6\)) 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. 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 ($C_{\text{max}}$), time in which $C_{\text{max}}$ is reached ($T_{\text{max}}$), 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
terminal phase ($t_{1/2}$). Furthermore, the relative bioavailability ($Fr \%$) of resveratrol was estimated by the following equation:

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

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

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

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

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

where $C_t$ is the plasma concentration of resveratrol at a time $t$, $k$ is the elimination rate constant of the polyphenol, $AUC\;0-t$ is the area under the resveratrol concentration vs. time curve from 0 to time $t$, and $AUC\;0-\infty$ is the area under the curve from 0 to infinity.

Linear regression analysis was applied to the *in vitro–in vivo* correlation plot and coefficient of determination ($R^2$) was calculated.

**Anti-inflammatory efficacy study**

**Animal model**

Four weeks-old (20-22 g) C57BL/6J female mice were purchased from Harlan (Barcelona, Spain) and housed in standard animal facilities (6 animals per cage with free access to food and drinking water). Housing conditions were maintained by controlled temperature and humidity and with 12 h on/off light cycles. Animals were allowed to acclimate for one week before the experiment.

*In vivo* anti-inflammatory studies were evaluated in an endotoxic shock model set up by intraperitoneal (ip) administration of LPS at a dose of 40 µg per mouse 29. Before administration, LPS was dissolved in PBS and vortexed during 30 min to complete 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**
Preparation and characterization of nanoparticles

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

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

In vitro release profile

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

The release profile of resveratrol from NPs was fitted to different mathematical release models. Using the Korsmeyer-Peppas equation, R² values were high (R²>0.96) and the exponent “n” value was 0.75±0.06. All of this suggests that the release of resveratrol from nanoparticles would be a combination of Fickian diffusion and erosion of the nanoparticle matrix. Under these circumstances, the Peppas-Sahlin model was applied and the erosion (Kₑ) and diffusion (Kₒ) constants were calculated (Kₒ = 0.08±0.02 h⁻¹/₂).
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 \( C_{\text{max}} \) 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 \( C_{\text{max}} \) 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
the different formulations to rats. The resveratrol AUC values from zein nanoparticle formulations were significantly higher ($p < 0.05$) than those observed for the polyphenol solution. Similarly, the resveratrol MRT was thirteen-times higher when administered in the form of zein nanoparticles than when solubilized in the PEG400:water oral mixture. Finally, the relative oral bioavailability of resveratrol when incorporated in nanoparticles was calculated to be 50% using zein nanoparticles. This value was significantly higher than the bioavailability obtained with the PEG400:water solution (2.6%).

Figure 4 shows the plasma concentration versus time profile of the resveratrol main metabolite (resveratrol-O-3-glucuronide) after the single administration of the polyphenol in the formulations tested. Interestingly, the profile of the plasma curves for both resveratrol and its metabolite were similar; however, the metabolite levels were always higher than for the polyphenol. When resveratrol was administered intravenously, the metabolite concentration reached 41.9 µg/mL ($C_{max}$) and, then, the metabolite levels decreased sharply. The AUC value was calculated to be 197 µg h/mL.

For the solution of resveratrol orally administered, the $C_{max}$ of the metabolite in plasma was found to be 2-times lower (22.1 µg/mL) than when administered by the iv route. In this case, the metabolite was only quantified in plasma during the first 8 h post-administration. The AUC value was calculated to be 104 µg h/mL; around half the i.v. solution one.

For nanoparticles, the metabolite was quantified during the first 24 hours after administration. In addition, the metabolite AUC data was around 342 µg h/mL for Rsv-NP-Z. This value was around three-times higher than with the resveratrol was administered as oral solution or intravenously.

**In vitro-in vivo correlations**

Figure 5 represents the relationship between the *in vitro* dissolution data (expressed 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 ºC. 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**
In the past zein was proposed as material for the preparation of nanoparticles due to its hydrophobic character, degradability, adherence properties and versatile processability. However, as zein possesses abundant non-polar amino acids, the dispersability of the resulting nanoparticles in an aqueous media (and, therefore, their potential applications) is a challenge. Recently, the use of citrate and phosphate salts was proposed to minimize this problem. In our case, lysine was added during the preparative process of nanoparticles. In this way, the resulting dry powder of zein nanoparticles was easily redispersed, yielding a homogeneous fine suspension (Table 1) after the addition of water and simple hand agitation.

Resveratrol-loaded zein nanoparticles (Rsv-NP-Z) displayed a mean size close to 300 nm and negative zeta potential. The resveratrol loading was of 80 µg/mg nanoparticles with an encapsulation efficiency of 80%. This payload is in line with values previously reported by using solid lipid nanoparticles, PLGA nanoparticles, or nanoemulsions. The release of resveratrol from zein nanoparticles was found to be pH-independent. In fact, this phenomenon would be a combination of both Fickian diffusion and erosion of the nanoparticle matrix (Peppas-Sahlin model). During the first hours of the release process, resveratrol molecules would mainly diffuse from the nanoparticles to the aqueous medium by Fickian diffusion. Later (3.5 h), the release of resveratrol would be mainly due to an erosion and/or relaxation process of the nanoparticle matrix. Interestingly, as a consequence of both phenomena, the amount of resveratrol released (at least during the first 8 h) is constant and approaches to a zero order kinetic.

Pharmacokinetic studies were carried out at a single dose of 15 mg/kg, comparable to those used in previous studies. The oral administration of a single dose of resveratrol as an aqueous suspension (Rsv-susp) to rats did not produce quantifiable levels of the polyphenol in plasma (Figure 3B). For the solution formulation, in a PEG400:water mixture (Rsv-sol), the plasma levels of the polyphenol were higher than for the suspension but they rapidly decreased and 6 h-post administration only traces
of resveratrol in plasma were detected. These findings are directly related with the extensive metabolism of resveratrol \(^{35}\). In fact, when administered orally, resveratrol (due to its lipophilic character) can rapidly enter into the enterocyte by passive diffusion \(^{36}\); although, it is highly metabolized to glucuronide and sulphate derivatives, which may be secreted back to the intestinal lumen through multidrug resistance protein 2 (MRP2) and BCRP \(^{37,38}\). This extensive biotransformation of resveratrol decreases circulation levels of free resveratrol and facilitates its excretion (in the form of conjugates) by the kidneys via urine \(^{14}\). Controversy remains about the physiological activity of metabolites or if they can act as resveratrol prodrugs. There are evidences that, at sufficient concentrations, resveratrol metabolites have biological activity in various tissues \(^{36}\). Nevertheless, there are also evidences that these compounds have no effects in some tissues \(^{39}\).

However, when resveratrol was administered after its encapsulation in zein nanoparticles, sustained and prolonged plasma levels of the polyphenol were observed for at least 24 h (Figure 3B) and its relative oral bioavailability was about 50% (Table 3), which is about 18-fold higher than the value observed for Rsv-sol (about 2.6%). This increased capability to promote the absorption and bioavailability of resveratrol by using zein nanoparticles would be related with its high hydrophobic character, that would offer a higher stability in vivo, and to the capability of this corn protein to develop mucoadhesive interactions within the gut mucus layer \(^{40}\). Thus, this characteristic would provide a longer residence in close contact with the intestinal epithelium and facilitating the establishment of a concentration gradient from the nanoparticulate matrix until the absorptive membrane. Interestingly, the fraction of resveratrol absorbed from zein nanoparticles correlated well with the percentage of the polyphenol released \textit{in vitro} (see Figure 5).

In previous studies, it has been reported that the oral bioavailability of resveratrol is almost zero \(^{34,41}\). In order to improve its absorption different strategies have been proposed such as the use of oral absorption enhancers (e.g. Tween 80, cyclodextrins)
or the employment of resveratrol derivatives. In this way, Kapetanovic and co-workers have reported an oral bioavailability of resveratrol (formulated as aqueous solution containing methylcellulose and Tween 80) close to 30% after the administration of a single dose of 50 mg/kg in rats. In the same work, the administration of the same resveratrol formulation at a dose of 150 mg/kg produced an oral bioavailability of 19%.

In another work, resveratrol trimethyl-ether administered orally in a solution formulated with randomly methylated-β-cyclodextrin (15 mg/kg) yielded a bioavailability of about 47%. More recently, the use of nanocarriers has also been proposed. Thus, in mice and using a dose of 50 mg/kg, the oral bioavailability of resveratrol when loaded in either Eudragit or chitosan/lecithin nanoparticles was calculated to be 39 and 61%, respectively. For solid lipid nanoparticles, the oral bioavailability of the polyphenol was found to be 8-fold higher than for a conventional solution of resveratrol. In our case, the resveratrol bioavailability was 18-fold higher when loaded in zein nanoparticles than when dissolved in the PEG400:water solution. Furthermore, zein nanoparticles offering sustained and prolonged levels of resveratrol in plasma provided a supplementary advantage when compared with other strategies.

Regarding the presence of the main metabolite (resveratrol-O-3-glucuronide) in the plasma of animals, the levels of this compound (measured as AUC) were higher when resveratrol was administered encapsulated in zein nanoparticles than when administered in the conventional solution both by iv route (about 1.7 times) or orally (around 3.3 times). This fact would be related with the slow release of the polyphenol from the nanoparticles (where protected from degradation) and a prolonged residence of nanoparticles in the gut mucosa due to their mucoadhesive properties. In other words, by using nanoparticles, more resveratrol and during a longer period would reach the circulation, counterbalancing the natural rapid metabolism of the drug.

Finally, we studied the anti-inflammatory activity of resveratrol when loaded in zein nanoparticles. Several in vitro and in vivo studies suggest that resveratrol inhibits the inflammatory response mediated by microbial stimuli, by inhibiting the transcription
factor NF-κB\(^{10,11}\). Therefore, we tested here the protective effect of encapsulated resveratrol against the inoculation of LPS in mice. LPS is present exclusively on the outer membrane of Gram negative bacteria, and consequently, it is one of the most strong alarm signals for the innate immune system, inducing in animals a pathophysiologic syndrome known as endotoxic shock. This syndrome is similar to sepsis shock syndrome that progress on multiple organ failure\(^{29,46}\), showing piloerection, hypothermia, shivering, tachycardia and lethargy. These symptoms are related with large amounts of released inflammatory mediators, such as TNF-α, NO and prostaglandin E2 (PGE2), where TNF-α play a central role as being the first one to be released\(^{10}\). In our experimental conditions, untreated mice challenged with LPS (positive control) displayed the highest decrease in rectal temperature and the highest TNF-α serum level. In contrast, Rsv-NP-Z administered daily during 7 days, were able to diminish endotoxic symptoms like hypothermia or piloerection and increase the movement of mice compared to those treated with resveratrol solution on daily basics (Figure 6, Table 3). Moreover, for animals treated with Rsv-NP-Z, TNF-α levels were lower than for controls; although the high variability of values abolished the statistical significance. These results appear to indicate that the presence of sustained high levels of resveratrol in plasma could be efficient to reduce the inflammatory mediators in endotoxic shock induced by LPS.

In summary, zein nanoparticles appear to be interesting carriers for the oral delivery of resveratrol. The polyphenol is released from this carrier by a combination of both diffusion and erosion of the nanoparticle matrix, providing higher and more prolonged plasma levels of resveratrol up to 48 h. Consequently, these nanocarriers significantly increased the oral bioavailability of resveratrol reaching a value close to 50%. The oral administration of these nanoparticles during one week to mice challenged with LPS protected them from the inflammatory symptoms and mediators of the endotoxic shock. Future studies should be performed to ascertain how this treatment modulates TNF-α
production in order to explore the potential use of Rsv-NP-Z as anti-inflammatory treatment.

Acknowledgements

This work was supported by the Regional Government of Navarra (Alimentos funcionales, Euroinnova call) and the Spanish Ministry of Science and Innovation and Gobierno de Navarra (ADICAP; ref. IPT-2011-1717-900000). Rebeca Penalva acknowledges the “Asociación de Amigos Universidad de Navarra” for the financial support.
References


Figure captions

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

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

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

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

Figure 5. Relationship between fractions dissolved in vitro vs. fraction absorbed in vivo of Resveratrol loaded into zein nanoparticles (Rsv-NP-Z). FRD (fraction of resveratrol 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.
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.
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.

<table>
<thead>
<tr>
<th></th>
<th>Size (nm)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>PDI</th>
<th>Zeta potential (mV)</th>
<th>Rsv loading (µg/mg NP)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>E.E. (%)&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP-Z</td>
<td>264 ± 2</td>
<td>0.07 ± 0.01</td>
<td>-46 ± 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rsv-NP-Z</td>
<td>307 ± 3</td>
<td>0.10 ± 0.01</td>
<td>-51 ± 0</td>
<td>80 ± 3</td>
<td>82 ± 4</td>
</tr>
</tbody>
</table>

<sup>a</sup> Determination of volume mean diameter by photon correlation spectroscopy

<sup>b</sup> Determination of resveratrol content by HPLC-UV

<sup>c</sup> 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)

<table>
<thead>
<tr>
<th>Route</th>
<th>Route</th>
<th>C&lt;sub&gt;max&lt;/sub&gt; (µg/mL)</th>
<th>T&lt;sub&gt;max&lt;/sub&gt; (h)</th>
<th>AUC (µg h/mL)</th>
<th>T ½ (h)</th>
<th>Cl (mL/h)</th>
<th>Vd (mL)</th>
<th>MRT (h)</th>
<th>Fr (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rsv iv.</td>
<td>iv</td>
<td>15.2± 5.18</td>
<td>0.1±0.0</td>
<td>10.4 ± 3.80</td>
<td>2.0±0.5</td>
<td>199 ± 89.8</td>
<td>569 ± 221</td>
<td>2.4±1.0</td>
<td>100</td>
</tr>
<tr>
<td>Rsv-sol</td>
<td>oral</td>
<td>0.20 ± 0.02</td>
<td>0.6±0.2</td>
<td>0.28 ± 0.13</td>
<td>0.3±0.2</td>
<td>387 ± 225</td>
<td>112 ±104</td>
<td>1.3±0.8</td>
<td>2.6</td>
</tr>
<tr>
<td>Rsv-susp</td>
<td>oral</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Rsv-NP-Z</td>
<td>oral</td>
<td>0.39 ± 0.11†</td>
<td>4.9 ± 3.1</td>
<td>5.17 ± 2.61†</td>
<td>5.5 ± 1.7</td>
<td>125 ± 41</td>
<td>909 ± 184</td>
<td>17.1 ± 7.1†</td>
<td>50.0</td>
</tr>
</tbody>
</table>

C<sub>max</sub>: peak plasma concentration; T<sub>max</sub>: 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.

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>T* decreased** &gt;2°C</th>
<th>Piloerection</th>
<th>Mobility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control -</td>
<td>0/6</td>
<td>-</td>
<td>Normal</td>
</tr>
<tr>
<td>Control +</td>
<td>6/6</td>
<td>+++</td>
<td>Very low</td>
</tr>
<tr>
<td>Rsv-Sol</td>
<td>4/6</td>
<td>++</td>
<td>Very Low</td>
</tr>
<tr>
<td>Rsv-NP-Z</td>
<td>1/6</td>
<td>+</td>
<td>Low</td>
</tr>
</tbody>
</table>

*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.
Anti-inflammatory effect (LPS endotoxic shock)