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Physicochemical and pharmacological characterization of α-tocopherol-loaded nano-emulsion system

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ABSTRACT

The main purpose of the present study is to develop a novel nano-emulsion (NE) formulation of α-tocopherol (α-TC) with enhanced oral bioavailability and pharmacological effects. Three NE formulations of α-TC at different loading amounts (10%, 30% and 50%) were prepared by a mechanochemical method. Physicochemical properties of NE formulations were characterized with a focus on the morphology by transmission electron microscopy (TEM), droplet size distribution and zeta-potential by dynamic light scattering (DLS), and long-term stability. According to the TEM images and DLS data, mean diameters of NE droplets ranged from 80 to 400 nm, in proportion to the amount of loaded α-TC. Although all NE formulations of α-TC were found to be negatively charged with the zeta-potential of ca −40 mV, NE formulations at α-TC content of 30% or higher exhibited severe aggregation of droplets in NE formulations during long-term storage. After oral administration of 10% α-TC-loaded NE formulation (30 mg α-TC/kg) in rats, higher α-TC exposure was observed with a 2.6-fold increase of bioavailability as compared to the control mixture of oil and α-TC. In streptozotocin-induced diabetic rats, oral administration of the α-TC-loaded NE formulation (30 mg α-TC/kg) exhibited a significant reduction of lipoperoxidant in several organs, especially the liver; however, the control mixture was less effective. With these findings, the NE approach might be efficacious to improve the oral bioavailability and anti-oxidative activities of α-TC.

1. Introduction

Vitamin E is an essential nutrient derived from various crops, such as barley, wheat, and soybean (Hakkarainen et al., 1984; Hall and Laidman, 1968; Ito et al., 2007). Vitamin E has several isomers, including α-, β-, γ-, and δ-tocopherols (Leth and Sondergaard, 1977), the activity and bioavailability of which vary depending on their structures and physicochemical properties. Among these isomers, *d*-α-tocopherol (α-TC) has the highest bioactivity, and its oral bioavailability is also higher than other vitamin E series (Hidiroglou and Karpinski, 1988). The α-TC prevents oxidative damage and lipid peroxidation in central and peripheral nervous systems (Scholz et al., 1997; Teranishi et al., 2001; Terrasa et al., 2009). Because of its promising therapeutic potential and safety, α-TC has been widely used as a functional food, in food additives, and cosmetics, as well as drugs (Biesalski, 2009; Jialal and Devaraj, 2005; Krol et al., 2000). However, the bioavailability of α-TC is not high enough and is sometimes affected by factors such as food consumption, lipid digestion, and micelle formation (Lodge et al., 2004; Sokol et al., 1989). Lower endogenous antioxidants with elevated lipid peroxidation levels have been identified as significant risk factors for oxidative stress-related diseases and diabetic complications (Baynes, 1991; Doi et al., 2001; Giugliano et al., 1996). Therefore, the development of α-TC formulations with improved bioavailability and pharmacodynamics might be a key consideration for the treatment of systemic oxidative stress.

Recently, attention has been drawn to nanoencapsulated systems (Mora-Huertas et al., 2010), showing high intracellular uptake, and improved stability and solubility of active substances. In particular, liquid nano-emulsion (NE) formulations have been used for the solubilization of poorly water-soluble drugs (Mora-Huertas et al., 2010; Shakeel and Faisal, 2010). NE strategies are often applied to lipophilic drugs, such as paclitaxel (Khandavilli and Panchagnula, 2007), primaquine (Singh and Vingkar, 2008), and saquinavir (Vyas et al., 2008). In addition to pharmaceutical substances, nano-emulsification of highly lipophilic neutraceuticals has been attempted in the production of beverages and liquid/gel foods (Boon et al., 2008; McClements et al., 2007). Previously, our

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Table 1
Composition of α-TC-loaded NE formulations.

Formulations	Composition (weight %)					
	α-Tocopherol	MCT	Glycerol	Decaglyceryl monooleate	Soybeanlecithin	Water
NE-1	10	5	55	12	3	15
NE-2	30	5	35	12	3	15
NE-3	50	5	15	12	3	15
Control mixture	10	90	–	–	–	–
Vehicle	–	15	55	12	3	15

group also developed a novel NE formulation of coenzyme Q_{10} using high-pressure homogenization (Hatanaka et al., 2008), and the NE formulation exhibited rapid and stable dispersion in water and improved oral bioavailability in rats. Thus, emulsifying techniques have been applied to a number of neutraceuticals, and the NE formulation approach might also be a promising delivery option for α-TC, possibly leading to improved clinical outcomes.

The main purpose of the present study was to develop a novel nano-emulsified formulation of α-TC with the aim of increasing pharmacokinetic and pharmacodynamics. In the present study, several liquid formulations of α-TC were prepared by a mechanochemical method. The physicochemical properties of the novel formulations were characterized with a focus on droplet size, zeta-potential, stability, and morphology. Pharmacokinetic profiling of α-TC after oral administration of NE formulation of α-TC or α-TC suspension in rats was carried out for comparison. In addition, experimental diabetic rats were prepared by intravenous administration of streptozotocin, and the anti-oxidative properties of newly developed NE formulation were evaluated in the diabetic rats.

2. Materials and methods

2.1. Chemicals

α-Tocopherol was purchased from MP Biomedicals (Solon, USA), medium chain triglyceride (MCT) was from Kao Corporation (Tokyo, Japan), decaglyceryl monooleate were from Taiyo Kagaku Co., Ltd. (Mie, Japan), soybean lecithin was from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan), glycerol was from Sakamoto Yakuhin Kogyo Co., Ltd. (Osaka, Japan), and storeptozotosin (STZ) was from Sigma Chemical Company (St. Louis, MO, USA).

2.2. Preparation of α-TC-loaded NE formulations

NE formulations, NE-1–3, listed in Table 1, were prepared using a mechanochemical method as previously described (Hatanaka et al., 2008). In short, the lipid phase, composed of α-TC and MCT, and the aqueous phase, composed of decaglyceryl monooleate, lecithin, glycerol and deionized water, were heated up to 80 °C separately. The lipid phase was added to the aqueous phase with processing by T.K. Robomix (PRIMIX Corporation, Osaka, Japan) at 9000 rpm for 15 min. The obtained primary emulsion was processed by a Microfluidizer (Mizuho Industry, Osaka, Japan) at 1000 kg/cm^2. The vehicle, α-TC-unloaded NE formulation, was prepared in the same manner. Control mixture, containing 10% (w/w) of α-TC, was also prepared by stirring MCT and α-TC at 40 °C, as a control of *in vivo* tests.

2.3. Dynamic light scattering (DLS)

The mean particle size and zeta-potential of the liposomes were measured by a dynamic light scattering, ELS-Z (Otsuka Electronics, Osaka, Japan). Prior to measurement, 0.5 g NE formulation was diluted with deionized water up to 50 mL. Diluted samples were analyzed at 25 °C. Scattered light was measured at an angle of 160°. The size distribution (polydispersity) was measured in terms of span factor defined as span = $(d_{90} - d_{10})/d_{50}$, where d_{10}, d_{50} and d_{90} are the particle diameters at 10%, 50% and 90% of the cumulative volume, respectively. Zeta-potential was calculated from the electrophoretic mobility by the Helmholtz–Smoluchowski equation. The determination was repeated three times per sample.

2.4. Laser diffraction (LD)

Droplet size by LD was analyzed using SALD-2100 (Shimadzu, Kyoto, Japan) equipped with a flow cell unit. Deionized water used as the dispersant. The data were evaluated using the volume distribution method. D99 value was calculated as the maximum value of droplet size distribution.

2.5. Transmission electron microscopy (TEM)

An aliquot (2 μL) of the NE solution diluted with deionized water was placed on a carbon-coated Formvar 200 mesh nickel grid. The sample was allowed to stand for 15–30 s, and then any excess solution was removed by blotting. The samples were negatively stained with 2% (w/v) uranyl acetate and allowed to dry. They were then visualized under a H-7600 transmission electron microscope (Hitachi, Japan) operating at 75 kV.

2.6. Pharmacokinetic property

2.6.1. Animals and drug administration

Male Wistar rats (Japan SLC, Shizuoka, Japan), weighing 260 ± 10 g, were housed two per cage in the laboratory with free access to diet and water, and maintained on a 12-h dark/light cycle in a room with controlled temperature (24 ± 1 °C) and humidity (55 ± 5%). All procedures used in the present study were conducted according to the guidelines approved by the Institutional Animal Care and Ethics Committee of the University of Shizuoka.

Rats were cannulated in the jugular vein and fasted for approximately 24 h before drug administration. For oral administration, the control mixture or NE formulation was suspended or dispersed in deionized water, and administered orally (30 mg α-TC/kg). For intravenous administration, NE formulation was dispersed in saline solution (1%), and injected (10 mg α-TC/kg). Each 150 μL blood sample was collected from the jugular vein via a cannula before and 2, 4, 6, 9, 12, 15, 18 and 24 h after administration. Plasma were separated by centrifugation at 10,000 rpm, 10 min, 4 °C and stored at below −20 °C until determination using HPLC-ECD analysis.

2.6.2. Plasma concentration of α-tocopherol

All analyses of α-TC were performed on a Shimadzu Prominence HPLC system (Shimadzu, Kyoto, Japan), which included the solvent delivery unit LC-20A with high-pressure flow-line selection valves, an auto sampler SIL-20A, and column oven CTO-20A, connected with LC solution software. α-TC was detected using an ESA Coulochem III 5010 detector (ESA, Inc., Chelmsford, MA) operating at an applied voltage of 0.5 V, as described by Lodge et al. (2000) with modifications. An CAPCELL PAK C18 MGII column (par-

ticle size: 5 μm, column size: Ø 3.0 mm × 150 mm; Shiseido, Tokyo, Japan) was used, and its temperature was maintained at 40 °C. Standards and samples were separated using a mobile phase consisting of 5% methanol and 95% acetonitrile containing 15 mmol/L $HClO_4$. The flow rate was set at 0.5 mL/min. Pharmacokinetic parameters were estimated using a non-compartment model method. Individual C_{max} values were calculated from actual plasma α-TC concentrations. AUC was calculated using the linear trapezoidal rule.

2.7. Pharmacological property

2.7.1. Diabetes induction and drug administration

Nine-week-old rats were injected with 30 mg/kg STZ into the tail vein, freshly dissolved in 0.1 mol/L citrate buffer. Blood was collected from the tail vein 72 h after STZ injection to determine blood glucose levels using ACCU-CHEK Comfort (Roche Diagnostics K.K., Tokyo, Japan). Two weeks after STZ challenge, vehicle composed of MCT, NE formulation and control mixture was administered orally (30 mg α-TC/kg). At 24 h after oral administration, the liver, kidney, and brain were collected after perfusion with saline, and each organ was stored at −80 °C after homogenization until analysis.

2.7.2. Thiobarbituric acid reactive substance (TBARS) concentration

TBARS concentrations were determined as malondialdehyde (MDA) using the fluorescence intensity of reactant with thiobarbituric acid (TBA), as described by Ohkawa et al. (1979). Briefly, the sample was homogenized with an adequate amount of 40 mmol/L phosphate buffer (pH 7.4) and diluted up to 500 μL. This solution was mixed 500 μL of 3% sodium dodecyl sulfate, 0.1 mol/L HCl 2 mL, 10% phosphotungstic acid 300 μL and 0.7% TBA solution 1 mL, and incubated at 100 °C for 45 min. After cooling, the solution was extracted with *n*-butanol 5 mL and fluorescence intensity was determined at 555 nm with excitation at 515 nm using a spectrofluorometer, RF-1500 (Shimadzu, Kyoto, Japan).

2.8. Statistical analysis

For statistical comparisons, one-way analysis of variance (ANOVA) with pairwise comparison by Fisher's least significant difference procedure was used. A *P* value of less than 0.05 was considered significant for all analyses.

3. Results and discussion

3.1. Preparation and characterization of α-TC-loaded NE formulations

In the present study, α-TC-loaded NE formulations were prepared by a mechanochemical method using a homomixer and microfluidizer. These NE formulations were composed of decaglyceryl monooleate and soybean lecithin as an emulsifier, MCT as an oil medium, and glycerol and water as aqueous media (NE-1–3; Table 1). NE formulations, containing 10% (w/w) and 30% (w/w) of α-TC, could be processed by a microfluidizer with ease. In contrast, NE-3, containing 50% (w/w) of α-TC, could be emulsified by only T.K. Robomix, but not by a microfluidizer, possibly due to its high viscosity. Although the appearance of NE-1 and -2 was found to be a translucent liquid with low viscosity and semi-translucent liquid with high viscosity, respectively, NE-3 was mainly observed as a poor fluidic paste. Differences in the transparency and viscosity of NE formulations might be attributable to the loading amount of the prepared α-TC formulations.

According to TEM images of NE formulations (Fig. 1), three prepared NE formulations seemed to be well dispersed in water. All

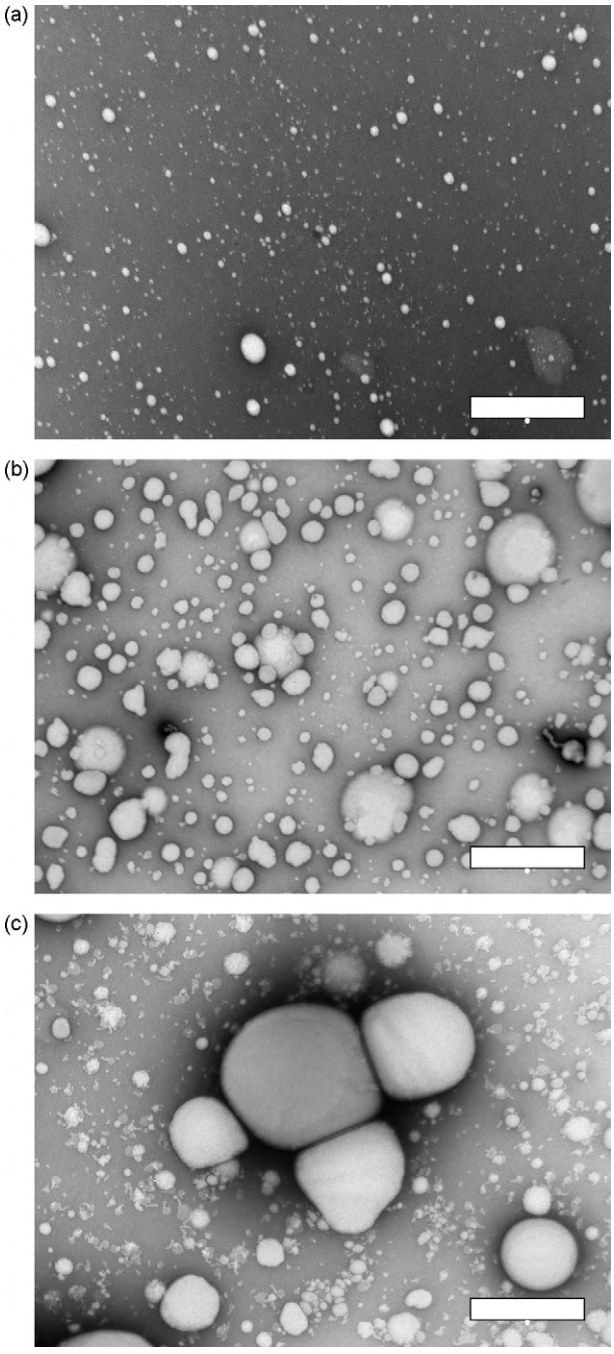

Fig. 1. Transmission electron microscopic images of α-TC-loaded NE formulations. NE formulations of α-TC, including NE-1 (A), NE-2 (B), and NE-3 (C), were dispersed in water. Bar represents 1 μm.

droplets in NE-1 were found to be fine enough, whereas there were some large droplets with a diameter >500 nm in NE-2. TEM image of NE-3 demonstrated the lower uniformity of emulsified droplets, as evidenced by some large droplets. On the basis of DLS analysis, the mean diameters of NE-1, -2, and -3 were calculated to be 85, 136, and 381 nm, respectively (Table 2). The span factors of NE-1, -2, and -3 were calculated to be 1.34, 1.34, and 2.35, respectively. Although NE-3 exhibited relatively high span factor, low span factors on NE-1 and -2 suggested low polydispersity and a narrow size distribution (Mondal et al., 2008). Size distribution of NE formulation was also measured by LD analysis since the measurability of DLS was highly limited in the over-micron range. D99 values in LD analysis demonstrated that 99% of all droplets were smaller than 0.15 μm

Table 2
Size distributions and zeta-potentials of α-TC-loaded NE formulations.

Formulations	Mean diameter (nm)	Span factor	zeta-potential (mV)	D99 (μm)*
NE-1	85 ± 0.8	1.34 ± 0.06	−41 ± 2.1	0.15 ± 0.00
NE-2	136 ± 1.9	1.34 ± 0.22	−39 ± 0.9	1.01 ± 0.02
NE-3	381 ± 15	2.35 ± 0.43	−36 ± 2.3	18.2 ± 0.19

Each NE formulation was dissolved in deionized water, and the mean diameter, span factor, and zeta-potential were determined by dynamic light scattering analysis. *: Maximum value of droplet size distribution in laser diffraction analysis. Data represent the mean ± SD of three independent experiments.

in NE-1, 1.01 μm in NE-2, and 18.2 μm in NE-3, respectively. The data were partly consistent with TEM experiments, suggesting that an excess amount of α-TC-loaded in NE formulations might affect the morphology of emulsion systems, possibly leading to decrease in long-term stability.

3.2. Stability of α-TC-loaded NE formulations

Generally, a desirable emulsion formulation should be easily dispersible in water and physically stable for a certain period (Washington, 1996). All NE formulations could be homogenously dispersed into water at the final α-TC concentration of 1 mg/mL. In the present study, the stability of NE formulations was investigated to choose a suitable NE system for α-TC. For the electrostatic stability of colloidal systems, the zeta-potential of dispersed droplets has been identified as an important factor (Washington, 1996). As shown in Table 2, all NE formulations were found to be negatively charged, as evidenced by a zeta-potential of ca. −40 mV, and they were similar to that of the α-TC-free vehicle (−41.2 mV). The NE formulations contained 3% soybean lecithin, consisting of free fatty acids and phospholipids. Negative zeta-potential values can be obtained due to the presence of negatively charged carboxyl groups in free fatty acids and phosphate groups in acidic phospholipids such as phosphatidylserine, phosphatidic acid, and phosphatidylinositol. The negative zeta-potential value would allow predicting good colloidal stability due to the high-energy barrier between particles (Mora-Huertas et al., 2010). Therefore, the soybean lecithin would be responsible for the electrostatic stabilization of fine emulsions, as well as the dispersing properties.

In a comparative stability study of NE formulations after dispersing into water, no phase separation was observed in all NE formulations stored at 40 °C, suggesting that α-TC molecules could be encapsulated into the fine emulsion dispersed in water. The water-dispersed NE-1 formulation was highly stable because any appearance changes, such as creaming and sedimentation, were negligible for at least 1 year (date not shown). In contrast, there was slight and gradual creaming in water-dispersed NE-2 and NE-3 formulations during only 7-day storage. After 1-year storage of water-dispersed NE formulations at room temperature, the mean particle sizes of NE-1, -2, and -3 were determined to be 90 ± 1, 500 ± 22, and 2,850 ± 272 nm, respectively (data not shown). Thus, marked increases of mean diameter were seen in both NE-2 and -3, and span factor of aged NE-3 was calculated to be as much as 8.09. These findings suggested that NE-1 was much more stable than NE-2 and -3, and this might be associated with the relatively low loading amount of α-TC in the NE-1. Thus, NE-1 was chosen as a suitable formulation of α-TC, and the pharmacokinetic and pharmacological properties were further clarified.

3.3. Pharmacokinetic behavior of NE formulation after oral administration

Observations on the high dispersion and stability of NE-1 formulation prompted us to clarify the possible improvement in intestinal absorption of α-TC, so the pharmacokinetic behaviors of α-TC formulations were assessed in rats. The plasma concentration–time profiles of α-TC in rats after oral administration of NE-1 and the control mixture of α-TC and MCT (30 mg α-TC/kg) are shown in Fig. 2. Plasma α-TC levels were found to be very low when the control mixture was administered orally, and C_{max} and AUC_{0-24} values were 1.4 ± 0.1 μg/mL and 23.9 ± 2.6 μg/mL h, respectively. In contrast, as compared to the control mixture, NE-1 exhibited significant improvement in bioavailability of α-TC ($P < 0.01$). Oral administration of NE-1 resulted in rapid elevation of plasma α-TC levels up to C_{max} 3.3 ± 0.2 μg/mL, and the AUC_{0-24} value was calculated to be 58.2 ± 6.0 μg/mL h. The bioavailability of the oral control mixture and NE-1 was calculated to be 3.9 ± 0.6 and 10.2 ± 1.0%, respectively. Thus, there appeared to be a ca. 2.6-fold enhancement in the bioavailability of α-TC using the nano-emulsion approach.

Generally, NE delivery systems have higher solubilization capacity compared with simple micellar solutions and suspensions, so that the NE strategies have been applied to several types of poorly soluble drugs and nutraceuticals (Mora-Huertas et al., 2010; Shakeel and Faisal, 2010). Although α-TC is practically insoluble in water (solubility at 25 °C: <0.1 μg/mL), α-TC in the present NE formulation could be dispersed as nano-sized fine droplets on the basis of size distribution analysis and TEM observation. Theoretically, the gastrointestinal absorption of poorly water-soluble drugs can be enhanced by emulsification, due to an increased surface area for direct contact with intestinal mucosa (Gursoy and Benita, 2004). Thus, the improved pharmacokinetic behavior of α-TC by NE approach might be partly attributed to the enhanced solubility and increased surface area. With these findings, taken together with the physicochemical properties, NE approaches would be effective for developing a water-soluble formulation of α-TC with improved systemic exposure.

3.4. Anti-oxidative effect of α-TC-loaded NE formulation in diabetic rats

STZ has been used to develop experimental diabetic animal models (Feillet-Coudray et al., 1999), since STZ acts as a potent DNA methylating agent and a free radical donor in pancreas, lead-

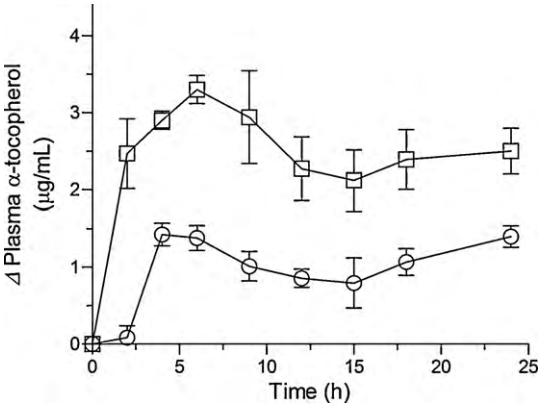

Fig. 2. Increase in plasma concentration of α-TC after oral administration of NE formulation (30 mg α-TC/kg). Control mixture of α-TC and MCT, ◯: NE-1, □: Each value represents the mean ± SE of 5–7 experiments.

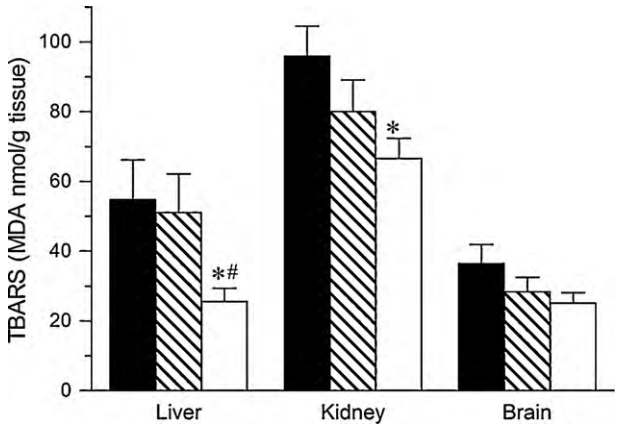

Fig. 3. Anti-oxidative effects of α-TC-loaded NE formulation in experimental diabetic rats. At 24 h after oral administration of α-TC formulations (30 mg α-TC/kg) in STZ-induced diabetic rats, concentrations of TBARS in the liver, kidney, and brain were determined. Filled bar, vehicle alone; hatched bar, control mixture of α-TC and MCT; and open bar, NE-1. Each value represents the mean ± S.E. of 8 rats. *: $P < 0.05$ with respect to vehicle alone; #: $P < 0.05$ with respect to the control mixture.

ing to poor glycemic control. In STZ-induced diabetic rats, severe oxidative stress was observed, as indicated by an increase of TBARS (Kakkar et al., 1998). Previously, Baydas and co-workers demonstrated that repeated injection of α-TC for several weeks resulted in the significant decrease of TBARS in STZ-induced diabetic rats (Baydas et al., 2002). Herein, the anti-oxidative effect of α-TC-loaded NE formulation was assessed on the basis of TBARS levels in STZ-induced diabetic rats. Two weeks after intravenous administration of STZ (30 mg/kg) in rats, there was marked increase in the blood glucose level up to ca. 500 mg/dL, reflecting the diabetic condition. No significant differences in the pharmacokinetic behavior of α-TC after oral administration of NE formulation were seen between normal and STZ-induced diabetic rats (data not shown). Oral administration of α-TC formulations to diabetic rats provided limited positive effects on glycemic levels since blood glucose levels in diabetic rats, control mixture- and NE-1-treated rats were determined to be 507.8 ± 25.4, 471.9 ± 22.3 and 477.3 ± 22.9 mg/dL, respectively. In spite of the limited anti-diabetic effects, α-TC formulations showed anti-oxidative effects in diabetic rats. According to the TBARS levels determined (Fig. 3), oral administration of the control mixture led to a slight reduction of oxidative stress in both the kidney and brain, and the control mixture was less effective in the liver. However, as compared to the diabetic rats, NE-1-treated rats exhibited the significant reduction of TBARS level by 53% in the liver ($P < 0.05$), 32% in the kidney ($P < 0.05$), and 31% in the brain ($P = 0.07$). The anti-oxidative effects of NE-1 formulation were found to be more potent than the control mixture in the liver ($P < 0.05$). The improvement in the anti-oxidative effects was consistent with the enhanced systemic exposure of α-TC after oral administration of NE-1. The variable anti-oxidative effects among rat tissues may be associated with the differences in the delivery of α-TC after oral administration of NE-1, which need to be clarified in further studies. These observations, taken together with physicochemical and pharmacokinetic profiles, suggest that the NE approach may be an effective for increase in the systemic exposure of oral α-TC, leading to management of the oxidative condition in some diseases.

4. Conclusion

In the present study, NE formulations, loading 10–50% of α-TC (w/w), were prepared using a mechanochemical method, and they were easily dispersed into water, forming nano-sized emulsion with a diameter of ca 80–400 nm. A stability study demonstrated that water-dispersed NE-1, containing 10% of α-TC, was the most stable in these formulations. After oral administration of NE-1 in rats, there appeared to be a 2.6-fold increase of systemic exposure of α-TC compared with the control mixture, an oil solution of α-TC. Oral administration of NE formulation resulted in the significant attenuation of oxidative stress in experimental diabetic model rats, as evidenced by the marked reduction of TBARS levels in the liver, kidney, and brain. In conclusion, NE formulation might be a promising delivery option for α-TC with the aim of improving oral bioavailability and anti-oxidative effects.

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