International Journal of Pharmaceutics 363 (2008) 206–213

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Nanoemulsions of an anti-oxidant synergy formulation containing gamma tocopherol have enhanced bioavailability and anti-inflammatory properties

Fonghsu Kuo [a], Balajikarthick Subramanian [a], Timothy Kotyla [a], Thomas A. Wilson [a], Subbiah Yoganathan [b], Robert J. Nicolosi [a,*]

[a] *Biomedical Engineering and Biotechnology Ph.D. Program, Center for Health and Disease Research, Division of NanoMedicine, University of Massachusetts Lowell, Lowell, MA 01854, United States*
[b] *Forsyth Institute, Boston, MA 02115, United States*

ARTICLE INFO

Article history:
Received 4 June 2008
Received in revised form 9 July 2008
Accepted 21 July 2008
Available online 31 July 2008

Keywords:
MicroFluidizer Processor®-based nanoemulsions
Anti-oxidant synergy formulation
Croton oil
Tocopherol

ABSTRACT

The present study investigated whether MicroFluidizer Processor®-based nanoemulsions of an antioxidant synergy formulation (ASF), containing delta, alpha and gamma tocopherol influenced inflammation and bioavailability in CD-1 mice. Croton oil was applied to all animals' right ear lobe to induce inflammation. Auricular thickness was measured after 2 and 6 h after the various treatments. The animal plasma and ear lobes were collected and frozen for bioavailability and cytokine analyses. The ASF nanoemulsions of alpha, delta, or gamma tocopherol significantly reduced auricular thickness compared to control (57, −57, and −71%, respectively) and blank nanoemulsion (−50, −50, −67%, respectively). Relative to the suspensions of ASF, only the nanoemulsion of ASF containing gamma tocopherol significantly reduced auricular thickness (−60%), whereas the 40% reduction with nanoemulsions of delta tocopherol compared to suspension was not statistically significant. Auricular concentrations of cytokines TNF-alpha and IL-1 alpha were significantly reduced in mice treated only with ASF nanoemulsions of gamma tocopherol compared to control (−53, −46%, respectively) and blank nanoemulsion (−52, −46%, respectively). Auricular thickness was significantly associated with tissue TNF-alpha ($r = 0.539$, $p < 0.001$) and IL-1 alpha concentrations ($r = 0.404$, $p = 0.01$). Bioavailability for gamma and delta was dramatically enhanced (2.2- and 2.4-folds) with the nanoemulsion compared to suspensions. Only the plasma gamma tocopherol concentration was significantly associated with auricular thickness ($r = -0.643$, $P = 0.001$). In conclusion, nanoemulsions of ASF containing gamma, alpha, and delta tocopherol, have enhanced anti-inflammatory properties and increased bioavailability, with gamma tocopherol, in particular compared to their suspensions.

1. Introduction

Inflammation is the body's first immune response when infected or irritated by external assault. Although inflammation is associated with the normal process of healing, the mechanism(s) involved in this process are quite complex, and when not well regulated, can result in inflammatory disease. In addition, clinical evidence has shown that chronic inflammation can contribute to the development of certain kinds of cancers (Coussens and Werb, 2002), neurodegenerative diseases (Stix, 2007) and atherosclerosis (Libby, 2002).

Vitamin E contains eight different isomers of tocopherols and tocotrienols that vary in degree of anti-oxidant and anti-inflammatory properties. All the various analogs of tocopherol and tocotrienol have similar structures on their chromanol ring with only the number of methyl groups on the chromanol ring differing between the alpha, beta, gamma, and delta isomers. In addition, the different bioavailability of each form is thought to explain the range of their biological activities and functions (Bieri and Evarts, 1974; Jiang et al., 2001; Valenzuela et al., 2002).

Alpha tocopherol is an important lipid peroxidation anti-oxidant and the predominant form of tocopherol found in mammalian circulation and tissues. Experimental data have shown that alpha tocopherol has ability to modulate several enzyme activities, such as protein kinase C, cyclooxygenase-2 (COX-2), phospholipase A2, and protein phosphatase 2A (Reiter et al., 2007; Zingg, 2007) which are involved in the signal transduction pathway of inflammation (Reiter et al., 2007). Although gamma tocopherol is the most abundant isomer in the American diet, its appearance

* Corresponding author at: 3 Solomont Way, Center for Health and Disease Research, University of Massachusetts Lowell, Lowell, MA 01854, United States. Tel.: +1 978 934 4501; fax: +1 978 934 2034.
E-mail address: nicolosi.robert@yahoo.com (R.J. Nicolosi).

0378-5173/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2008.07.022

in the blood stream (bioavailability) is limited reportedly, in part, as a result of reduced retention time of gamma tocopherol in the body when compared to alpha tocopherol (Jiang et al., 2001). Recent data from experimental studies suggest that gamma tocopherol can act as an anti-inflammatory agent by reducing the synthesis of prostaglandin E_2, through the inhibition of cyclooxygenases such as COX-2 (Wolf, 2006).

Previously, our lab has reported that an anti-oxidant synergy formulation (ASF), which contained alpha tocopherol exerts synergistic effects on neuroblastoma cells by providing neuroprotection against oxidative stress, reducing cellular proliferation, and promoting differentiation in a series of in vitro experiments (Amy et al., 2003). As a result of this study, we hypothesized that (a) this ASF formulation might have other biological indications (anti-inflammation) and also whether the individual tocopherol isomers in the ASF formulation were different, as it relates to inflammatory activity (b) the bioavailability and efficacy of this tocopherol component of ASF formulation might similarly be enhanced by altering the nano-delivery system, in particular, from the self-assembly polyethylene glycol (PEG)-based polymer/solvent nanospheres reported in our initial studies of tocopherol (Shea et al., 2005) to the present nanoemulsion formulation utilizing Microfluidizer Processor® Technology. These possible outcomes were enhanced by our recent reports that agents delivered by our MicroFluidizer Processor®-based nanoemulsion system showed increased anticancer indications (Kuo et al., 2007; Tagne et al., 2008) and bioavailability (Kotyla et al., 2008).

Much attention has focused on developing alternative drug delivery systems, nanoscale, in particular, in order to improve the bioavailability of poorly water soluble or hydrophobic compounds which could lead to greater efficacy. Compared to most other formulations, emulsion preparations, composed of oil, surfactant, and water are associated with less toxicity and increase bioavailability and efficacy of the various encapsulants. For example, microemulsions containing micron-sized particles (thousands of nanometers in size) increase water solubility and bioavailability of nitrendipine, prednisolone, and betamethasone (Kawakami et al., 2002; Lawrence and Rees, 2000). A logical extension of these published reports are that, since nanoemulsions have particle sizes usually less than or slightly greater than 100 nm and have increased surface-to-volume ratio, they would be expected to significantly enhance bioavailability and efficacy as reported by others, (Kumar et al., 2004; Shafiq et al., 2007) and from our own laboratory with encapsulants consisting of such diverse compounds as ASF (Kuo et al., 2007), tamoxifen (Tagne et al., 2008), and tocopherol (Kotyla et al., 2008).

The objectives of the current study were to utilize the inflammatory CD-1 mouse model which we have previously reported on (Yoganathan et al., 2003) to (a) compare the anti-inflammatory properties of ASF containing different tocopherol isomers and (b) evaluate whether nanoemulsion formulations of ASF containing different tocopherol isomers will affect the bioavailability and efficacy compared to their control, empty nanoemulsions and suspension counterpart.

2. Materials and methods

2.1. Suspension and nanoemulsion preparations

Alpha, delta, and gamma tocopherol isomers, sodium pyruvate, polysorbate 80 and phosphatidyl choline were purchased from Sigma–Aldrich Inc. (St. Louis, MO). Suspensions of these ingredients were prepared in the following manner: phosphatidyl choline and tocopherol isomers were dissolved in ethanol and a mixture of sodium pyruvate and HPLC-grade water were added and then homogenized for 30 s (Polytron Model PT 10/35, Brinkmann Instr., Westbury, NY). For the preparation of nanoemulsions, phosphatidyl choline and tocopherol isomers were dissolved in soybean oil at 50 °C and a mixture of sodium pyruvate, polysorbate-80 and HPLC-grade water were added and then homogenized for 30 s. This preparation was subsequently subjected to a Microfluidizer® Processor-Model M-110EH (Microfluidics, Newton, MA) as we have described previously (Kotyla et al., 2008; Kuo et al., 2007; Tagne et al., 2008). The final concentrations of each of the components of the ASF suspensions and nanoemulsions were 0.54 mg/mL phosphatidyl choline, 2.6 mg/mL sodium pyruvate, and 23 mg/mL of alpha, delta, or gamma tocopherol. Both the suspensions and nanoemulsions were then mixed with vanishing cream (PCCA, Houston, TX 77099) in a 1:1 ratio before application as we have described previously (Kotyla et al., 2008; Kuo et al., 2007).

The particle sizes of the ASF suspensions and nanoemulsions were determined by dynamic laser light scattering using the Malvern Zetasizer-S instrument (Malvern Instruments Inc., Southborough, MA) at 25 °C. Each sample was diluted immediately before measurement with distilled water to avoid multiple light scattering effects. A previous report has indicated that the dilution of samples did not change the particle size distribution (Muller et al., 2002). The mean hydrodynamic diameter (DH) was calculated from the Strokes–Einstein equation. The range of particle sizes which can be measured by the Zetasizer is from 0.6 to 6000 nm.

2.2. Morphological evaluation of the nanoemulsion

Characterization of the nanoemulsion morphology included the use of transmission electron microscopy (TEM) [Philips EM400T]. A modification of the method for the emulsion sample preparation for TEM analysis (Desai et al., 2007; Zhao et al., 2006) was used for this study. The nanoemulsion was diluted in a ratio of 1:200 with distilled/deionized water and one drop of the dilution was subsequently taken and placed into the carbon attached Vinylec films (Ernest F. Fullam Inc., Latham, NY). The excess liquid was removed with filter paper and allowed to stand for 10 min. The grid was then stained with 4% osmium tetroxide (Sigma–Aldrich, St. Louis, MO) and allowed to dry for 5 min. The sample was then placed in a vacuum chamber for 30 min prior to TEM analysis.

2.3. Animal models

Male CD-1 mice, 6 weeks of age (Charles River Laboratories, Wilmington, MA) with an average body weight ranging from 25–29 g were used for this study. Upon arrival, mice were group-housed in polycarbonate cages with bedding for 1 week prior to the study, to allow them to adapt to standard laboratory conditions (temperature 23 ± 0.5 °C, relative humidity 50% and 12/12/light/darkcycle). The CD-1 mouse is one of the preferred choices as an inflammatory mouse model due to its greater responsiveness to croton oil-induced inflammation when compared to Balb/C or C57BL6/J strain (Koyuncu et al., 1999). In addition, the CD-1 strain lacks T4 cells which are part of the anti-inflammatory mechanism of the immune system, making them a useful inflammatory model (Politis and Dmytrowich, 1998; Trotta et al., 1996; Yoganathan et al., 2003). Experiments using the CD-1 mouse were performed according to the guidelines of the Institutional Animal Care and Use Committee of the University of Massachusetts Lowell.

2.4. Experimental protocol

Forty-five animals were divided into nine groups containing five animals per group. Initially, all mice were anesthetized with 150 μL of a solution containing ketamine (100 mg/mL), xylazine

(20 mg/mL) and sterile water in a ratio of 1:1:4 just prior to measurement of right auricular thickness using an electronic digital caliper (VWR International, West Chester, PA). The pro-inflammatory inducing agent Croton oil (2%) (Sigma, St. Louis, MO) was dissolved in acetone and 50 μL was applied to the inner surface of the right auricular of the appropriate animal groups in order to induce an inflammatory response. Auricular thickness measurements were repeated after a 2-h period of induction of inflammation, as we have previously reported (Yoganathan et al., 2003). The following treatments were subsequently applied topically to the inflamed right auricular of the mice: (1) control-no treatment, (2) blank nanoemulsion, (3) vanishing cream, (4) nanoemulsion of ASF (alpha tocopherol), (5) nanoemulsion of ASF (gamma tocopherol), (6) nanoemulsion of ASF (delta tocopherol), (7) ASF suspension (alpha tocopherol), (8) ASF suspension (gamma tocopherol), and (9) ASF suspension (delta tocopherol). Each treatment applied was gently rubbed until it was no longer visible and this application process will insure that all treatments were completely applied and no waste occurred. Besides, each ASF tocopherol isomer-treated group contained the same amount of tocopherol (1.15 mg/isomer). Six hours after treatment, auricular thickness measurements were repeated and blood was collected from each anesthetized animal via retro-orbital sinus into heparinized capillary tubes. Plasma was harvested from blood after centrifugation at 1500 × *g* at 4 °C for 20 min. The animals were then euthanized and right ear lobes collected, immediately frozen in liquid nitrogen and then stored at −80 °C for subsequent plasma tocopherol and tissue cytokine analyses. Auricular thickness differences were calculated by subtracting the auricular thickness obtained after 6 h post-treatment from the initial thickness measurement obtained at the beginning (0 h) of the experiment.

2.5. Cytokine measurements

Auricular tissue was mixed with 1 mL lysis buffer containing 10 mM HEPES buffer, 0.01 μM Gentamicin, 0.5% Triton X-100, and 1% protease inhibitor cocktail (Sigma, St. Louis, MO). Tissue homogenates were prepared utilizing the Polytron Model PT 10/35 homogenizer (Brinkmann Instr., Westbury, NY). The tissue homogenates were centrifuged at 15,000 rpm for 15 min and the supernatant was collected and stored at −80 °C. The protein concentration was measured by the Bradford assay (Sigma, St. Louis, MO) and an equivalent amount of protein was utilized for IL-1α and TNF-α measurements using a colorimetric enzyme immunoassay (ELISA) (Endogen, Cambridge, MA; Bio Source Intl., Camarillo, CA).

2.6. Plasma tocopherol measurements

Plasma alpha, gamma, and delta tocopherols were determined by high-performance liquid chromatography (HPLC) using a modification of the procedure of Handelman et al. (1985) and as we have previously described (Kotyla et al., 2008). Briefly, plasma samples were mixed with 150 μL water and 250 μL isopropanol containing BHT (50 ug/mL). Samples were then extracted with 3 mL hexane and ether (1:1). After 60 s of vortex mixing, supernatants were collected and dried down under N_2. Subsequently, the residues were reconstituted with 60 μL ethanol containing BHT (50 ug/mL) and analyzed by HPLC using an Agilent 1100 series (Quantum Analytics Inc., Foster City, CA 94404) equipped with a micro-vacuum degasser, diode array and multiple wavelength detector, and fluorescence detector. For the HPLC measurement, 10 μL of sample was injected onto a Zorbax Rx-C18 (4.6 mm × 25 cm) column and 100% methanol was used as a mobile phase at a flow rate of 0.5 mL/min. Tocopherols were monitored with a fluorescence detector at excitation and emission wavelengths of 290 and 330 nm, respectively, and the UV detector was set at 295 nm. Both external standards of each isomer and an internal standard of tocol (92.2 mg/mL) were used to measure plasma concentrations of each isomer.

2.7. Statistical analysis

Sigma Stat software (Jandel Scientific, San Rafael, CA) was used to analyze the experimental data. A repeated-measures one-way analysis of variance (RM ANOVA) followed by the Student–Newman–Keuls separation of means was used to determine group differences for auricular thickness. An ANOVA followed by the Student–Newman–Keuls separation of means was used to determine group differences for tissue cytokines and plasma tocopherol concentrations. Correlations between auricular thickness, tissue IL-1α and TNF-α concentrations, and plasma concentrations of isomers were performed using Pearson's product moment correlation coefficient. All values were expressed as mean ± standard error mean (S.E.M.) and $p < 0.05$ was established as the criterion for significance.

3. Results

3.1. Measurement of particle sizes of the ASF formulations

The various ASF formulations were subjected to dynamic laser light scattering particle size analysis. The average particle sizes of the ASF suspensions were 843.0 ± 9.2 nm, 758 ± 10.5 nm and 673 ± 13.6 nm (mean ± S.E.M.; $n = 3$ measurements) for alpha, delta, and gamma tocopherol, respectively) (Table 1). In comparison, for the ASF nanoemulsions containing different tocopherol isomers, the dramatically reduced average particle sizes were 56.6 ± 0.5 nm, 51.1 ± 0.5 nm and 42.3 ± 0.2 nm (mean ± S.E.M.; $n = 3$ measurements) for alpha, delta, and gamma tocopherol, respectively) (Table 1). In addition, morphology of gamma tocopherol-encapsulated nanoemulsion was characterized by TEM and the particle size was approximately the same as the diameters measured by the dynamic light scattering instrument. TEM images indicated that all the nanoemulsion particles were well distributed and spherical (Fig. 1). TEM was also performed for the ASF nanoemulsions containing the different tocopherol isomers and were not different from each other (data not shown).

3.2. The effect of ASF with different tocopherol isomers on auricular thickness

Auricular thickness measurements for the mice receiving the ASF suspensions of the tocopherol isomers containing alpha (0.10 ± 0.03 mm), gamma (0.10 ± 0.02 mm), or delta (0.10 ± 0.01 mm) tocopherol were not significantly reduced compared to control (0.14 ± 0.01 mm) (Table 2). In contrast, compared to (a) control, animals receiving the ASF nanoemulsions containing alpha, delta, or gamma tocopherol had significantly reduced auricular thickness (−57, −57, and −71%, respectively), (b) cream (−50, −50, and −67%, respectively), and (c) blank nanoemulsion (−50, −50, −67%, respectively) (Table 2). Relative to the suspension preparations of ASF, only the nanoemulsion of ASF containing gamma tocopherol significantly reduced auricular thickness (−60%, $p < 0.03$) whereas the 40% reduction of the nanoemulsion of delta tocopherol compared to its suspension preparation did not reach statistical significance ($p = 0.1$) (Table 2).

No significant differences were observed between nanoemulsions containing the different isomers, although the nanoemulsion of ASF containing gamma tocopherol was associated with a further

Table 1
Physical and chemical properties of the anti-oxidant synergy formulations (ASF) containing different tocopherol isomers

Formulations	Composition	Polydispersity index (PDI)	Particle size (nm)
Blank nanoemulsion	SO + P_{80}	0.230 ± 0.007	53.2 ± 0.8
ASF suspension (AT)	SP + PC + AT	0.603 ± 0.071	843.0 ± 9.2
ASF nanoemulsion (AT)	SO + P_{80} + SP + PC + AT	0.287 ± 0.001	56.6 ± 0.2
ASF suspension (GT)	SP + PC + GT	0.721 ± 0.050	673 ± 13.6
ASF nanoemulsion (GT)	SO + P_{80} + SP + PC + GT	0.275 ± 0.003	42.3 ± 0.2
ASF suspension (DT)	SP + PC + DT	0.665 ± 0.050	758 ± 10.5
ASF nanoemulsion (DT)	SO + P_{80} + SP + PC + DT	0.272 ± 0.005	51.1 ± 0.5

Abbreviations: SP = sodium pyruvate, PC = phosphatidyl choline, SO = soybean oil, P_{80} = polysorbate 80, AT = alpha tocopherol, GT = gamma tocopherol, and DT = delta tocopherol
Values are means ± S.D., $n = 3$.

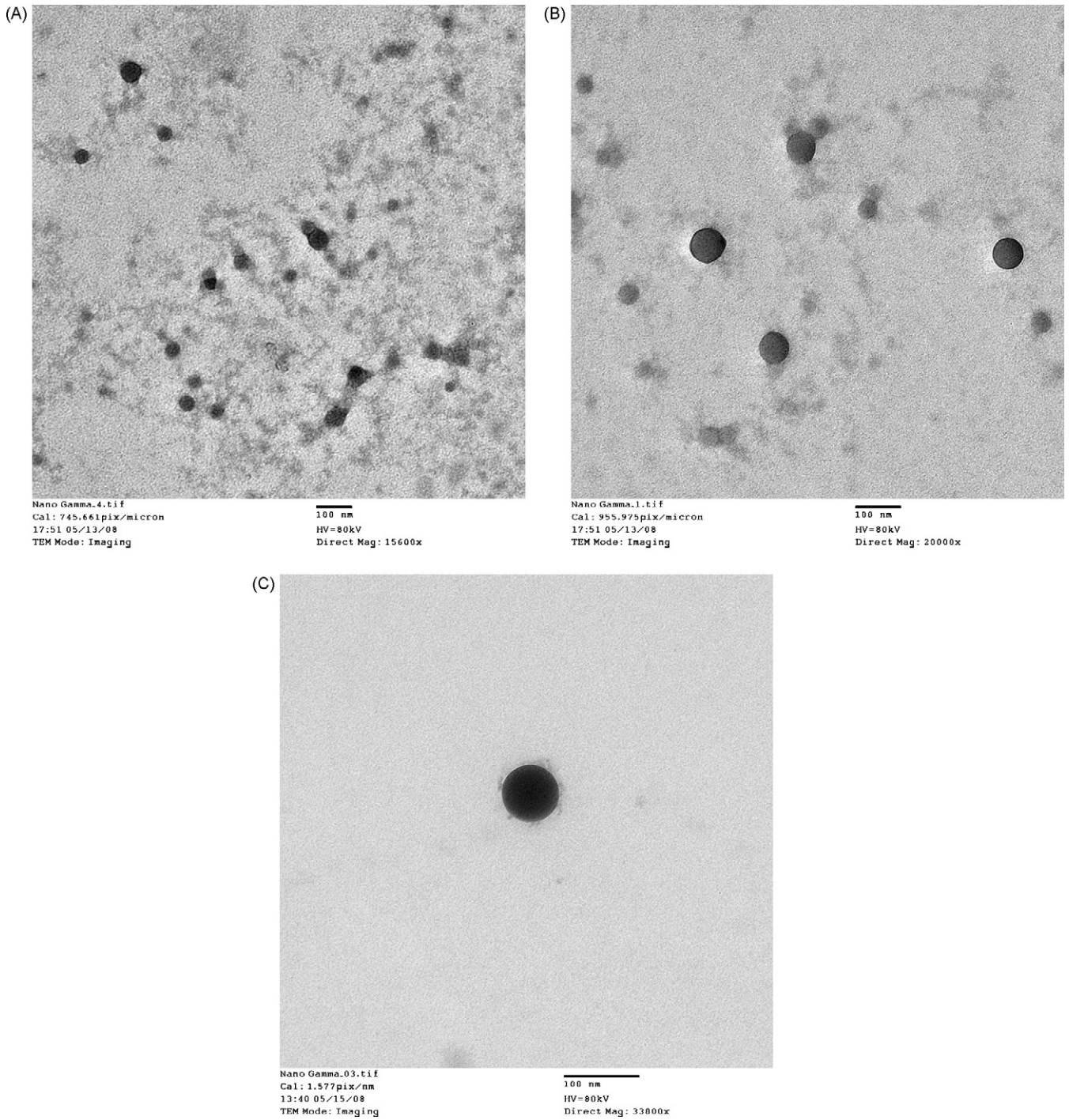

Fig. 1. TEM. illustrates the appearance and particle size of gamma tocopherol at different magnifications. (A) Mag. = 15600x (B) Mag. = 20000x (C) Mag. = 33000x. Since the other TEM's for the other tocopherol isomers were essentially identical, they are not included for sake of brevity.

Table 2
Auricular thickness (mm), tissue TNF-a (pg/mg of tissue), and tissue IL-la (pg/mg of tissue) concentrations in mice 6 h after treatment

Treatment	Auricular thickness	TNF-α	IL-lα
Control	0.14 ± 0.01a	19.1 ± 3.16a	428.3 ± 28.3ac
Cream	0.12 ± 0.02ac	19.0 ± 1.49a	485.6 ± 53.8a
Blank nanoemulsion	0.12 ± 0.01a	18.5 ± 2.25a	479.5 ± 43.8a
ASF suspension (AT)	0.10 ± 0.03ab	13.3 ± 1.48ab	318.4 ± 45.2abc
ASF nanoemulsion (AT)	0.06 ± 0.0lbc	10.8 ± 0.59b	421.8 ± 48.9ac
ASF suspension (GT)	0.10 ± 0.02a	10.0 ± 1.93b	281.9 ± 35.5bc
ASF nanoemulsion (GT)	0.04 ± 0.02b	8.9 ± 0.75b	229.7 ± 29.9b
ASF suspension (DT)	0.10 ± 0.01ab	13.6 ± 1.32ab	320.9 ± 29.7abc
ASF nanoemulsion (DT)	0.06 ± 0.0lbc	11.6 ± 0.78b	295.6 ± 32.7bc

Values are means ± S.E.M., $n = 5$. Values in a column not sharing a common letter are significantly different at $p < 0.05$. Abbreviations: AT = alpha tocopherol, GT = gamma tocopherol, and DT = delta tocopherol.

non-statistically significant reduction in auricular thickness (−33%) compared to the nanoemulsions containing alpha or delta tocopherol, The animals treated with the blank nanoemulsion or cream did not result in significant lower ear lobe thickness compared to control.

3.3. The effect of ASF on tissue TNF-α and IL-1α concentrations

Compared to control, cream- and blank nanoemulsion-treated mice, tissue concentrations of TNF-α from auricular homogenates was significantly reduced in the mice treated with ASF nanoemulsions containing alpha (−43, −43, and −42%, respectively), delta (−39, −39, and −38%, respectively), or gamma (−53, −53, and −52%, respectively) tocopherol (Table 2). The mice treated with the ASF suspension containing gamma tocopherol also had reduced tissue TNF-α concentrations compared to control (−47%), cream (−47%), and blank nanoemulsion (−46%) while the other ASF suspensions (alpha and delta) did not (Table 2). No significant differences in TNF-α concentration as a function of tocopherol isomer were observed between the various nanoemulsions of ASF and their corresponding suspensions of ASF for tissue TNF-α concentrations. The animals treated with the blank nanoemulsion or cream did not result in significantly lower tissue TNF-α concentrations compared to control.

The tissue concentration of IL-1α was significantly reduced in the mice treated with ASF nanoemulsions containing gamma tocopherol compared to control (−46%) but not for the mice treated with ASF nanoemulsions containing alpha or delta tocopherol (Table 2). The tissue concentration of IL-1α was significantly reduced in the mice treated with ASF nanoemulsions containing gamma and delta tocopherol compared to cream (−53 and −39%, respectively) and blank nanoemulsion (−46 and −30%, respectively) but not for the mice treated with ASF nanoemulsions containing alpha tocopherol (Table 2). Also, the tissue concentration of IL-1α was significantly reduced in the mice treated with ASF nanoemulsions containing gamma tocopherol compared to the mice treated with ASF nanoemulsions containing alpha tocopherol (−46%) (Table 2). The tissue IL-1α concentration was also significantly reduced in the mice treated with the ASF suspension containing gamma tocopherol compared to cream (−42%) and blank nanoemulsion (−41%) (Table 2). No significant differences were observed between the various nanoemulsions of ASF and their corresponding suspensions of ASF for tissue IL-1α concentrations. The animals treated with the blank nanoemulsion or cream did not result in significantly lower tissue IL-1α concentrations compared to control.

Auricular thickness was significantly correlated with tissue IL-1α ($r = 0.404$, $p = 0.01$) (Fig. 2) and TNF-α ($r = 0.539$, $p < 0.001$) (Fig. 3) concentrations, when comparing all treatment groups together.

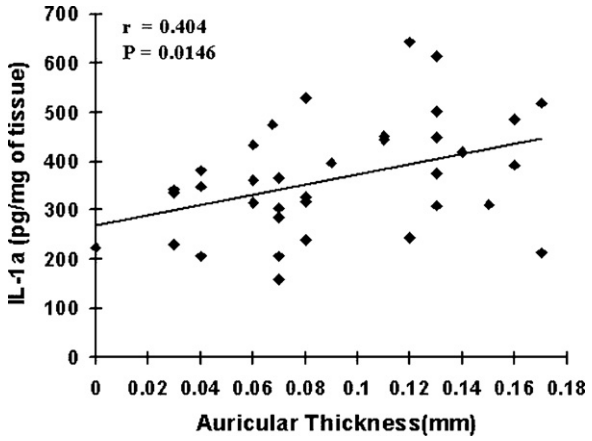

Fig. 2. Significant correlation between auricular concentration of IL-1α and auricular thickness.

3.4. The bioavailability of tocopherol isomers observed at 6 h after treatment

While both the ASF suspensions and nanoemulsions containing gamma or delta tocopherol significantly raised their blood concentrations compared to control, only the ASF nanoemulsions containing gamma or delta tocopherol significantly enhanced their plasma concentrations 2.2- and 2.4-fold, respectively, compared to the ASF suspensions containing these isomers (Table 3). ASF suspensions and nanoemulsions containing alpha tocopherol did not significantly raise plasma alpha tocopherol concentrations in

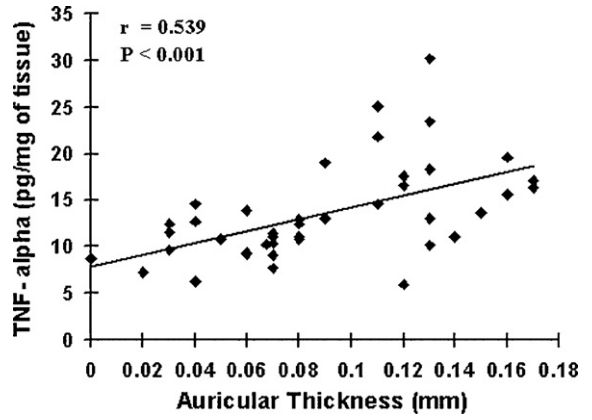

Fig. 3. Significant correlation between auricular concentration of TNFα and auricular thickness.

Table 3
Plasma alpha, gamma, and delta tocopherol concentrations (ng/mL) in mice 6 h after treatment

Treatment	Alpha tocopherol	Gamma tocopherol	Delta tocopherol
Control (Croton oil)	1426.7 ± 242.5a	95.2 ± 30.1a	ND
Cream	1317.1 ± 470.8a	156.8 ± 22.1a	ND
Blank nanoemulsion	1538.1 ± 212.2a	164.6 ± 39.7a	ND
ASF suspension (AT)	1937.0 ± 498.9a	177.1 ± 31.3a	ND
ASF nanoemulsion (AT)	1952.1 ± 315.6a	132.8 ± 33.5a	ND
ASF suspension (GT)	1087.4 ± 306.6a	519.9 ± 189.3b	ND
ASF nanoemulsion (GT)	1419.1 ± 164.5a	1153.1 ± 41.1c	ND
ASF suspension (DT)	1256.1 ± 32.9a	113.9 ± 50.5a	108.8 ± 48.4a
ASF nanoemulsion (DT)	1305.8 ± 136.6a	125.0 ± 22.8a	262.1 ± 35.8b

Values are means ± S.E.M., $n = 5$. Values in a column not sharing a letter are significantly different at $p < 0.05$. ND = none detected. Abbreviations: AT = alpha tocopherol, GT = gamma tocopherol, and DT = delta tocopherol.

the mice following application compared to control. Furthermore, the concentration of gamma tocopherol in a nanoemulsion-treated mice reached 60% of plasma alpha tocopherol concentration (Table 3).

Additionally, only the concentration of plasma gamma tocopherol was significantly associated with reduced auricular thickness ($r = -0.643$, $P = 0.001$) for all treatment groups (Fig. 4).

4. Discussion

The current study has found that particle size is a very critical factor in decreasing inflammation and increasing transdermal permeability of certain tocopherol isomers. Smaller particle size is considered to be advantageous since the interfacial area of encapsulated compounds into the aqueous phase is maximized (Pan et al., 2002; Vila et al., 2005). According to previous experiments, the results indicated that Microfluidizer Processor®-based nanoemulsion system significantly increase the bioavailability of transdermally applied delta tocopherol when compared to micron-sized emulsion preparation of delta tocopherol, this data suggested that the increase bioavailability of delta tocopherol in the nanoemulsion preparation was contributed from the size reduction of emulsion particles (Kotyla et al., 2008). In the current study, mouse auricular thickness was not significantly reduced compared to control with suspensions of ASF, which had particle sizes ranging from 680–850 nm. However, ASF nanoemulsions, which had particle sizes of approximately 50 nm, significantly reduced auricular thickness compared to control. Since there were 15-fold differences in particle size, the total surface area of the nanoemulsions should be dramatically greater than the suspensions further enhancing the delivery efficiency of ASF. This finding is in agreement with a previous study where small emulsion particles improved drug efficacy that was most likely due to the lipid and surfactant composition and physiochemical characteristics of the small emulsion particles (Constantinides et al., 2000). Surfactants have been utilized as one of the components in various microemulsion formulations for enhancing the penetration of methotrexate (Trotta et al., 1996) and ketoprofen (Rhee et al., 2001). Other studies have shown polysorbate 80, one particular surfactant, was able to accelerate hydrocortisone and lidocaine permeation (Sarpotdar and Zatz, 1986). Also, the formulation of a polysorbate 80-based microemulsion for the delivery of the poorly soluble drug nitrendipine caused a rapid increase in plasma nitrendipine concentration in vivo (Kawakami et al., 2002). In the current study, polysorbate 80 was used as a nonionic surfactant and we are suggesting that the enhanced bioavailability and efficacy seen with some of our nanoemulsion preparations may not only contribute from the small particle size but also the use of the surfactant.

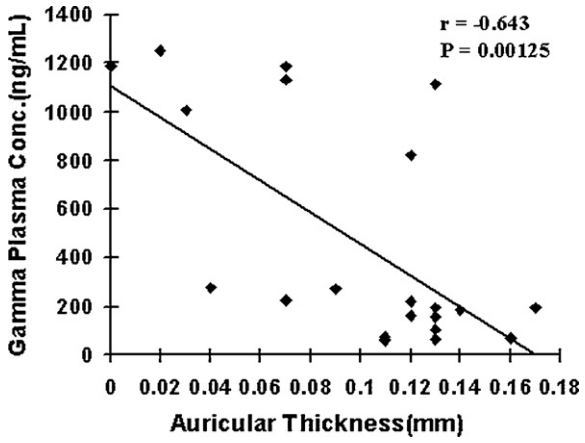

Fig. 4. Significant correlation for only the plasma levels of gamma tocopherol and auricular thickness in animals receiving the nanoemulsion preparation of gamma tocopherol.

Both micro- and nanoemulsion formulations have been used as excellent vehicles to solubilize lipophilic drugs and significantly improve bioavailability (Ghosh et al., 2006; Khandavilli and Panchagnula, 2007; Shafiq et al., 2007; Shen and Zhong, 2006; Tiwari and Amiji, 2006). In the current study, the bioavailability and therefore the plasma level of the ASF nanoemulsion containing gamma tocopherol was 2.2-fold greater than the ASF suspension ($p < 0.05$) with the equivalent amount of initial gamma tocopherol concentration. In addition, the increase in gamma tocopherol bioavailability from the nanoemulsion, as shown by an increase in plasma gamma tocopherol concentration, resulted in a significant reduction of auricular thickness ($r = -0.643$, $p = 0.001$) in the mice. ASF nanoemulsions containing delta tocopherol also dramatically increased delta tocopherol concentrations (2.4-fold) in plasma compared to ASF suspensions containing delta tocopherol ($p < 0.05$) in mice. Although delta tocopherol concentrations increased as much as gamma tocopherol in the nanoemulsion groups compared to the suspensions, auricular thickness was decreased only 28% by the nanoemulsion of ASF containing delta tocopherol, compared to the nanoemulsion of ASF containing gamma tocopherol which reduced auricular thickness by 43%. It is possible that this difference in efficacy may be associated with different potencies of each tocopherol isomer.

Plasma alpha tocopherol concentrations did not significantly change with ASF nanoemulsion containing alpha tocopherol following treatment compared to plasma concentrations in the mice treated with the corresponding suspension or any other treatment. It is possible that since plasma alpha tocopherol concentrations are very high in all the mice to begin with and may have reached a maximum amount in the blood, that treatment with the nanoemulsion

or suspension of ASF containing alpha tocopherol will not produce a further increase in plasma concentrations but possibly only an increase in tissue/storage concentrations. Unfortunately, tissue concentrations of tocopherol were not measured in the current study.

The production of nanoemulsions enhanced the anti-inflammatory properties of ASF containing gamma tocopherol. Experimental evidence has shown that gamma tocopherol is superior to alpha tocopherol in trapping reactive nitrogen oxide species during inflammation and exhibits stronger inhibition of lipid peroxidation than alpha tocopherol (Christen et al., 1997). In addition, gamma tocopherol and its metabolite (2,7,8-trimethyl-2-(b-carboxyethyl)-6-hydroxychroman (CEHC)) significantly inhibited COX-2 activity (Jiang et al., 2000) and decreased the accumulation of prostaglandin E_2 at the site of inflammation (Jiang and Ames, 2003). With regard to ASF nanoemulsions containing alpha and delta tocopherol, although we observed improved anti-inflammatory properties, relative to controls and empty nanoemulsion. ASF, nanoemulsions containing gamma tocopherol had 60% greater anti-inflammatory properties than their suspension counterpart. This finding suggests that ASF nanoemulsions containing different tocopherol isomers possess different effects on immune function.

Both TNF-α and IL-1α are pro-inflammatory cytokines that act as important mediators which can initiate the expression of other cytokines, recruit macrophages and neutrophils, and provoke inflammatory responses at the site of inflammation. TNF-α and IL-1α can activate nuclear factor-kappa B (NFκB), which is a primary transcription factor regulating the gene expression of other pro-inflammatory cytokines including TNF-α and IL-1α themselves. This regulatory mechanism is thought to be involved in the pathogenesis of several inflammatory diseases, such as asthma and rheumatoid arthritis (Li and Verma, 2002). Studies have shown that TNF-α antagonists have successfully inhibited TNF-α expression and have been used for the treatment for rheumatoid arthritis (Criscione and St Clair, 2002). Research has also showed that large amounts of IL-1α is synthesized in rheumatoid synovial tissue (Robak and Gladalska, 1997), which may be another pathway for anti-inflammatory therapy in rheumatoid arthritis. In the current study, nanoemulsions containing gamma tocopherol decreased the expression of TNF-α and IL-1α significantly. This finding suggests that an ASF nanoemulsion containing gamma tocopherol might have the potential against inflammatory diseases by regulating TNF-α and IL-1α expression. The observed correlations between TNF-α, IL-1α and auricular thickness, suggests that tocopherol, especially gamma, encapsulated ASF nanoemulsions can reduce auricular thickness by attenuating the amount of TNF-α and/or IL-1α produced at the site of inflammation. The production of TNF-α and/or IL-1α is relatively lower in the ASF suspension treated groups when compared to control, but this was only significantly lower for TNF-α production with gamma tocopherol. These results may suggest that ASF, as an suspension or nanoemulsion, containing gamma tocopherol may possess greater anti-inflammatory properties compared to alpha or delta tocopherol by reducing TNF-α and IL-1 production in this mice model.

In conclusion, this study indicates that ASF nanoemulsion preparations containing gamma, alpha, or delta tocopherol have (a) enhanced anti-inflammatory properties in CD-1 mice that are associated with decreased auricular thickness, and the production of TNF-α and/or IL-1α; (b) increased bioavailability compared to suspensions of these compounds; (c) ASF nanoemulsions or suspensions containing gamma tocopherol show greater anti-inflammatory properties compared to ASF nanoemulsions or suspensions containing alpha or delta tocopherol and (d) the plasma concentration of gamma tocopherol delivered as a nanoemulsion approaches amounts that are usually found for the more bioavailable alpha tocopherol.

Acknowledgements

We acknowledge Animal Facility Manager Sheryl Perry for her technical assistance and Maureen Faul of the Center for Health and Disease Research for her administrative assistance. These studies were partially supported by Microfludics Corp., Newton, MA 02464

References

Amy, L.H., EnkeledaF N., Nicolosi, R., Shea, T.B., 2003. An antioxidant formulation that induces differentiation of neuroblastoma in culture. Neurosci. Res. Commun. 33, 73–75.
Bieri, J.G., Evarts, R.P., 1974. Vitamin E activity of gamma-tocopherol in the rat, chick and hamster. J. Nutr. 104, 850-851-857.
Christen, S., Woodall, A.A., Shigenaga, M.K., Southwell-Keely, P.T., Duncan, M.W., Ames, B.N., 1997. Gamma-tocopherol traps mutagenic electrophiles such as NO(X) and complements alpha-tocopherol: physiological implications. Proc. Natl. Acad. Sci., U. S. A. 94, 3217–3222.
Constantinides, P.P., Lambert, K.J., Tustian, A.K., Schneider, B., Lalji, S., Ma, W., Wentzel, B., Kessler, D., Worah, D., Quay, S.C., 2000. Formulation development and antitumor activity of a filter-sterilizable emulsion of paclitaxel. Pharm. Res. 17, 175–182.
Coussens, L.M., Werb, Z., 2002. Inflammation and cancer. Nature 420, 860–867.
Criscione, L.G., St Clair, E.W., 2002. Tumor necrosis factor-alpha antagonists for the treatment of rheumatic diseases. Curr. Opin. Rheumatol. 14, 204–211.
Desai, A., Vyas, T., Amiji, M., 2007. Cytotoxicity and apoptosis enhancement in brain tumor cells upon coadministration of paclitaxel and ceramide in nanoemulsion formulations. J. Pharm. Sci..
Ghosh, P.K., Majithiya, R.J., Umrethia, M.L., Murthy, R.S., 2006. Design and development of microemulsion drug delivery system of acyclovir for improvement of oral bioavailability. AAPS PharmSciTechnol. 7, 77.
Handelman, G.J., Machlin, L.J., Fitch, K., Weiter, J.J., Dratz, E.A., 1985. Oral alpha-tocopherol supplements decrease plasma gamma-tocopherol levels in humans. J. Nutr. 115, 807–813.
Jiang, Q., Ames, B.N., 2003. Gamma-tocopherol, but not alpha-tocopherol, decreases proinflammatory eicosanoids and inflammation damage in rats. FASEB J. 17, 816–822.
Jiang, Q., Christen, S., Shigenaga, M.K., Ames, B.N., 2001. Gamma-tocopherol, the major form of vitamin E in the US diet, deserves more attention. Am. J. Clin. Nutr. 74, 714–722.
Jiang, Q., Elson-Schwab, I., Courtemanche, C., Ames, B.N., 2000. Gamma-tocopherol and its major metabolite, in contrast to alpha-tocopherol, inhibit cyclooxygenase activity in macrophages and epithelial cells. Proc. Natl. Acad. Sci. U.S.A. 97, 11494–11499.
Kawakami, K., Yoshikawa, T., Hayashi, T., Nishihara, Y., Masuda, K., 2002. Microemulsion formulation for enhanced absorption of poorly soluble drugs II. In vivo study. J. Control Rel. 81, 75–82.
Khandavilli, S., Panchagnula, R., 2007. Nanoemulsions as versatile formulations for paclitaxel delivery: peroral and dermal delivery studies in rats. J. Invest. Dermatol. 127, 154–162.
Kotyla, T., Kuo, F., Moolchandani, V., Wilson, T., Nicolosi, R., 2008. Increased bioavailability of a transdermal application of a nano-sized emulsion preparation. Int. J. Pharm. 347 (1–2), 144–148.
Koyuncu, H., Berkarda, B., Baykut, F., Soybir, G., Alatli, C., Gul, H., Altun, M., 1999. Preventive effect of hesperidin against inflammation in CD-1 mouse skin caused by tumor promoter. Anticancer Res. 19, 3237–3241.
Kumar, R., Chen, M.H., Parmar, V.S., Samuelson, L.A., Kumar, J., Nicolosi, R., Yoganathan, S., Watterson, A.C., 2004. Supramolecular assemblies based on copolymers of PEG600 and functionalized aromatic diesters for drug delivery applications. J. Am. Chem. Soc. 126, 10640–10644.
Kuo, F., Kotyla, T., Wilson, T., Kifle, L., Panagiotou, T., Gruverman, I., Tagne, J.B., Shea, T., Nicolosi, R., 2007. A nanoemulsion of an anti-oxidant synergy formulation reduces tumor growth rate in neuroblastoma-bearing nude mice. J. Exp. Ther. Oncol. 6, 129–135.
Lawrence, M.J., Rees, G.D., 2000. Microemulsion-based media as novel drug delivery systems. Adv. Drug Deliv. Rev. 45, 89–121.
Li, Q., Verma, I.M., 2002. NF-kappaB regulation in the immune system. Nat. Rev. Immunol. 2, 725–734.
Libby, P., 2002. Inflammation in atherosclerosis. Nature 420, 868–874.
Muller, R.H., Radtke, M., Wissing, S.A., 2002. Solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) in cosmetic and dermatological preparations. Adv. Drug Deliv. Rev. 54, S131–S155.
Pan, Y., Zheng, J.M., Zhao, H.Y., Li, Y.J., Xu, H., Wei, G., 2002. Relationship between drug effects and particle size of insulin-loaded bioadhesive microspheres. Acta Pharmacol. Sin. 23, 1051–1056.

Politis, M.J., Dmytrowich, A., 1998. Promotion of second intention wound healing by emu oil lotion: comparative results with furasin, polysporin, and cortisone. Plast. Reconstr. Surg. 102, 2404–2407.
Reiter, E., Jiang, Q., Christen, S., 2007. Anti-inflammatory properties of alpha- and gamma-tocopherol. Mol. Aspects Med. 28, 668–691.
Rhee, Y.S., Choi, J.G., Park, E.S., Chi, S.C., 2001. Transdermal delivery of ketoprofen using microemulsions. Int. J. Pharm. 228, 161–170.
Robak, T., Gladalska, A., 1997. Cytokines in rheumatoid arthritis. Postepy. Hig. Med. Dosw. 51, 621–636.
Sarpotdar, P.P., Zatz, J.L., 1986. Evaluation of penetration enhancement of lidocaine by nonionic surfactants through hairless mouse skin in vitro. J. Pharm. Sci. 75, 176–181.
Shafiq, S., Shakeel, F., Talegaonkar, S., Ahmad, F.J., Khar, R.K., Ali, M., 2007. Development and bioavailability assessment of ramipril nanoemulsion formulation. Eur. J. Pharm. Biopharm. 66, 227–243.
Shea, T.B., Ortiz, D., Nicolosi, R.J., Kumar, R., Watterson, A.C., 2005. Nanosphere-mediated delivery of vitamin E increases its efficacy against oxidative stress resulting from exposure to amyloid beta. J. Alzheimers Dis. 7, 297–301.
Shen, H., Zhong, M., 2006. Preparation and evaluation of self-microemulsifying drug delivery systems (SMEDDS) containing atorvastatin. J. Pharm. Pharmacol. 58, 1183–1191.
Stix, G., 2007. A malignant flame: understanding chronic inflammation, which contributes to heart disease, Alzheimer's and a variety of other ailments, may be a key to unlocking the mysteries of cancer. Sci. Am. 297, 60–67.
Tagne, J.B., Kakumanu, S., Ortiz, D., Shea, T., Nicolosi, R.J., 2008. A nanoemulsion Formulation of tamoxifen increases its efficacy in a breast cancer cell line. Mol. Pharm. 5, 280–286.
Tiwari, S.B., Amiji, M.M., 2006. Improved oral delivery of paclitaxel following administration in nanoemulsion formulations. J. Nanosci. Nanotechnol. 6, 3215–3221.
Trotta, M., Pattarino, F., Gasco, M.R., 1996. Influence of counter ions on the skin permeation of methotrexate from water–oil microemulsions. Pharm. Acta Helv. 71, 135–140.
Valenzuela, A., Sanhueza, J., Nieto, S., 2002. Differential inhibitory effect of alpha-, beta-, gamma-, and delta-tocopherols on the metal-induced oxidation of cholesterol in unilamellar phospholipid-cholesterol liposomes. J. Food Sci. 67, 2051–2055.
Vila, A., Sanchez, A., Evora, C., Soriano, I., McCallion, O., Alonso, M.J., 2005. PLA-PEG particles as nasal protein carriers: the influence of the particle size. Int. J. Pharm. 292, 43–52.
Wolf, G., 2006. How an increased intake of alpha-tocopherol can suppress the bioavailability of gamma-tocopherol. Nutr. Rev. 64, 295–299.
Yoganathan, S., Nicolosi, R., Wilson, T., Handelman, G., Scollin, P., Tao, R., Binford, P., Orthoefer, F., 2003. Antagonism of croton oil inflammation by topical emu oil in CD-1 mice. Lipids 38, 603–607.
Zhao, X., Liu, J.P., Zhang, X., Li, Y., 2006. Enhancement of transdermal delivery of theophylline using microemulsion vehicle. Int. J. Pharm. 327, 58–64.
Zingg, J.M., 2007. Modulation of signal transduction by vitamin E. Mol. Aspects Med. 28, 481–506.