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Development of topical nanoemulsions containing the isoflavone genistein

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We have recently described the incorporation of genistein into topical nanoemulsions. This study describes the physicochemical properties and the genistein permeation profile from these nanoemulsions. Formulations composed of egg lecithin, medium chain triglycerides (MCT) or octyldodecanol (ODD) and water were prepared by spontaneous emulsification. Irrespective of the oil core employed (MCT or ODD), this procedure yielded monodisperse emulsions with mean droplet sizes in the range of 230–280 nm. The addition of genistein in the oil phase, before emulsification, did not alter the properties of nanoemulsions. The amount of genistein incorporated in both formulations was close to 100% (1 mg/mL). Solubility and DSC experiments suggested that egg-lecithin may play an important role on the incorporation of genistein in nanoemulsions. Genistein permeation from formulations was assessed using pig ear skin in Franz type diffusion cells. The overall results showed a slow permeation profile for genistein from both nanoemulsions. Such results open interesting perspectives for the topical administration of genistein.

1. Introduction

In recent years, the effect of soybean isoflavones on the protection of skin photoaging and photocarcinogenesis has been extensively investigated. Several studies have addressed the activity of genistein, one of the most abundant soybean isoflavones (Messina et al. 1994; Wei et al. 1995, 2002; Kang et al. 2003; Afaq and Mukhtar 2006; Moore et al. 2006). Wei and co-workers (2003) have recently reviewed the skin effect of topically administered genistein. It was shown that genistein inhibits both ultraviolet-induced skin carcinogenesis and photodamage in mouse skin and also protects human skin against ultraviolet-induced photodamage.

Despite the significant interest on topical application of genistein, only few reports have described its skin permeation profile from formulations. Most of them have focused on the percutaneous absorption of genistein aiming at its transdermal delivery. Minghetti et al. (2006) have recently evaluated *in vitro* the effect of different permeation enhancers on genistein permeation through the human skin using modified Franz type diffusion cells. Polyethyleneglycol 400 was considered as the most effective vehicle for the transdermal delivery of genistein based on its higher permeation flux. Vänttinen and Möravcova (2001) have previously suggested the percutaneous absorption of genistein from an olive oil suspension through human skin by evaluating the isoflavone levels found in plasma and urine.

The incorporation of flavonoid aglycones such as genistein in well-accepted hydrophilic vehicles is frequently limited due to solubility reasons. Recently, the design of nanoemulsions as a vehicle for the topical delivery of poorly-soluble drugs has been receiving increasing attention (Piemi et al. 1999; Fernandez et al. 2000; Alves et al. 2005; Fasolo et al. 2007). Nanoemulsions are fine oil-in-water (o/w) dispersions in which the poorly-soluble drugs can be dissolved in the oil core and/or adsorbed at the o/w interface. In fact, the incorporation of drugs in such systems might increase their skin permeation rate and enhance the topical effect due to prolonged residence time in the uppermost skin layers due to the large surface area and low surface tension of the oil droplets (Klang et al. 1998; Benita 1999; Bouchemal et al. 2004).

In this context, the development of topical nanoemulsions containing genistein is currently under study by our research group. Recently, we have developed and validated a liquid chromatography (LC) method for genistein assay in topical nanoemulsions (Silva et al. 2007). Thus, the purpose of this study was to investigate the main physicochemical properties of the topical nanoemulsions as well as to evaluate genistein permeation profile from these formulations.

2. Investigations, results and discussion

2.1. Physico-chemical properties

Table 1 shows the physico-chemical properties of medium chain triglycerides (MCT) and octyldodecanol (ODD)-based emulsions obtained by spontaneous emulsification.

Table 1: Physico-chemical properties of nanoemulsions

	MCT	MCT/GEN	ODD	ODD/GEN
Droplet size (nm)	229 ± 16	255 ± 21	270 ± 18	278 ± 12
Viscosity (cP)	1.45 ± 0.02	1.54 ± 0.04	1.73 ± 0.03	1.81 ± 0.04
ζ-potential (mV)	−41.3 ± 8.0	−40.3 ± 8.1	−39.9 ± 5.2	−40.3 ± 4.9
Association efficiency (%)[a]	–	99.5 ± 0.6	–	97.0 ± 0.3

[a] For an initial concentration of 1 mg/ml

Such oils were chosen in order to evaluate their effect on both genistein incorporation into nanoemulsions and skin permeation profile. The mean droplet sizes varied from 229 to 278 nm, and the viscosity from 1.45 to 1.81 cP, in agreement with our previous data (Silva et al. 2007; Fasolo et al. 2007). Concerning ζ-potential determinations, nanoemulsions displayed negative values due to the presence of negatively-charged phospholipids in egg lecithin composition such as phosphatidic acid, as described in previous literature (Yang and Benita 2000; Li and Tian 2002).

The development and validation of a separation method based upon ultrafiltration allowed us to estimate the genistein content as well as the amount of free genistein in the external water phase of nanoemulsions (Silva et al. 2007). The association efficiency was estimated by the difference between the total and free genistein determined in the water phase. Whatever the nature of the oil core (MCT or ODD) was, the encapsulation efficiency was close to 100% for an initial concentration of 1 mg/ml (Table 1). These results could be attributed to the high affinity of this flavonoid aglycone for the lipid composition of the inner phase of nanoemulsions. In fact, the reported octanol-water partition coefficient of genistein is 3.04 (Rohthwell et al. 2005), which is typical for a lipophilic compound.

The incorporation of genistein (at 1 mg/ml) did not alter either physicochemical or morphological properties of nanoemulsions. The data presented in Table 1 show only a slight increase in the mean droplet size and viscosity of nanoemulsions compared to the blank ones. Transmission electron microscopy (TEM) investigations of the oil droplets (Fig. 1) showed the typical appearance of an o/w emulsion with droplets displaying mean size in the range of 200–300 nm, which is in agreement with the results obtained in photon correlation spectroscopy (PCS) experiments. A higher contrast at the interface of oil droplets can be related to the affinity of uranyl acetate used for negative staining to interface components.

In order to have a better insight about the location of genistein in nanoemulsions, its solubility in both MCT and ODD oils was determined. The solubility of genistein was 235.3 ± 2.2 μg/ml and 137.9 ± 2.5 μg/ml for MCT and ODD, respectively. These results clearly indicate the low solubility of genistein in both oils and suggest a possible role of egg-lecithin on genistein incorporation in nanoemulsions. In this way, DSC experiments were performed to investigate the effect of egg-lecithin on the thermal profile of genistein. Figure 2 shows the results of DSC analysis of genistein (raw material), egg-lecithin and the physical mixture of egg-lecithin/genistein. The DSC curve indicated that genistein exhibits a single endothermic peak at 302 °C which was attributed to its melting point, in agreement with previous reports (Motlekar et al. 2006). The curve for pure egg-lecithin shows slight endothermic peaks between 125 and 225 °C. Genistein/egg-lecithin mixture displayed different thermograms compared to pure egg-lecithin or genistein. Moreover, the disappearance of the peak corresponding to genistein melting endotherm was observed at 302 °C, suggesting some interactions between phospholipids of egg-lecithin and genistein. Such interactions could be related to the high incorporation efficiency of genistein into nanoemulsions (Table 1) even over the genistein solubility in the oil core. Taking into account these results, genistein is supposed to be distributed between the oil core and the interface of nanoemulsions, associated to phospholipid molecules of egg-lecithin.

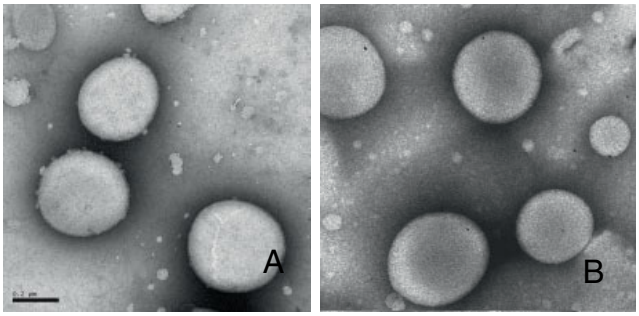

Fig. 1: TEM micrographs of nanoemulsions TCM/GEN [A] and ODD/GEN [B]

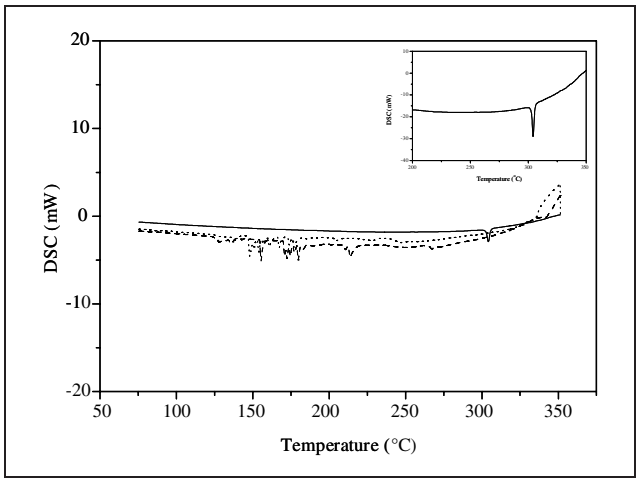

Fig. 2: DSC thermograms of genistein (straight), egg-lecithin (dot) and genistein/egg-lecithin mixture (dash). Insert is genistein thermogram magnified 10-fold

Table 2: Permeation parameters in percutaneous release of genistein from nanoemulsions

Parameters[a]	Intrinsic	ODD/GEN	TCM/GEN
Flux (μg/cm²/h)	13.22 ± 2.71	3.59 ± 0.50	2.89 ± 0.88
Lag time (h)	1.69 ± 0.40	1.90 ± 052	2.66 ± 0.24

[a] The results express the mean ± S.D. of three independent experiments

2.2. Skin permeation

In vitro skin permeation profile of genistein was performed using ear pig skin mounted in Franz diffusion cells. In a first step, genistein permeation from a volatile reservoir was evaluated. Figure 3A shows the amount of permeated genistein per unit surface area (μg/cm^2) *versus* time (h). After 8 h, the total amount of genistein permeated was approximately 60 μg/cm^2 with a flux of 13 μg/cm^2/h. However, even if the use of a volatile reservoir is an interesting approach to evaluate the intrinsic percutaneous flux in order to know the drug ability to allow skin penetration, these findings may be taken with caution due to a possible influence of the solvent on the pig ear skin before its evaporation (Mayorga et al. 1996).

Irrespective of the nature of the oil core, genistein skin permeation from nanoemulsions was slower than that observed from the volatile reservoir (Fig. 3B). In fact, the incorporation of genistein in nanoemulsions seems to increase the lag time and reduce the flux values of genistein in skin (Table 2). These findings could be attributed to the interactions between genistein and phospholipids from egg-lecithin, as previously discussed, which in turn increases the affinity of genistein for the vehicles. Our results also indicate that the total amount of genistein permeated from nanoemulsions composed by ODD as oil core was significantly higher (21 μg/cm^2) than that observed for MCT formulations (15 μg/cm^2). In fact, the flux value was approximately 1.4-fold higher for ODD-nanoemulsions, as can be seen in Table 2. This observation could be related to the fact that ODD can act as a better skin permeation enhancer for genistein. However, since genistein solubility in MCT was higher than in ODD, the hypothesis that a fraction of genistein remained soluble in the oil core of nanoemulsions, reducing its partitioning in the skin, may not be ruled out.

In conclusions, this study demonstrates the potential suitability of nanoemulsion as a topical delivery system for genistein. Whatever the oil core used, the physico-chemical studies suggested the role of lecithin on the incorporation of genistein into nanoemulsions. Considering the slower genistein flux observed from nanoemulsions when compared to the intrinsic flux, such a system could be useful for the local skin administration.

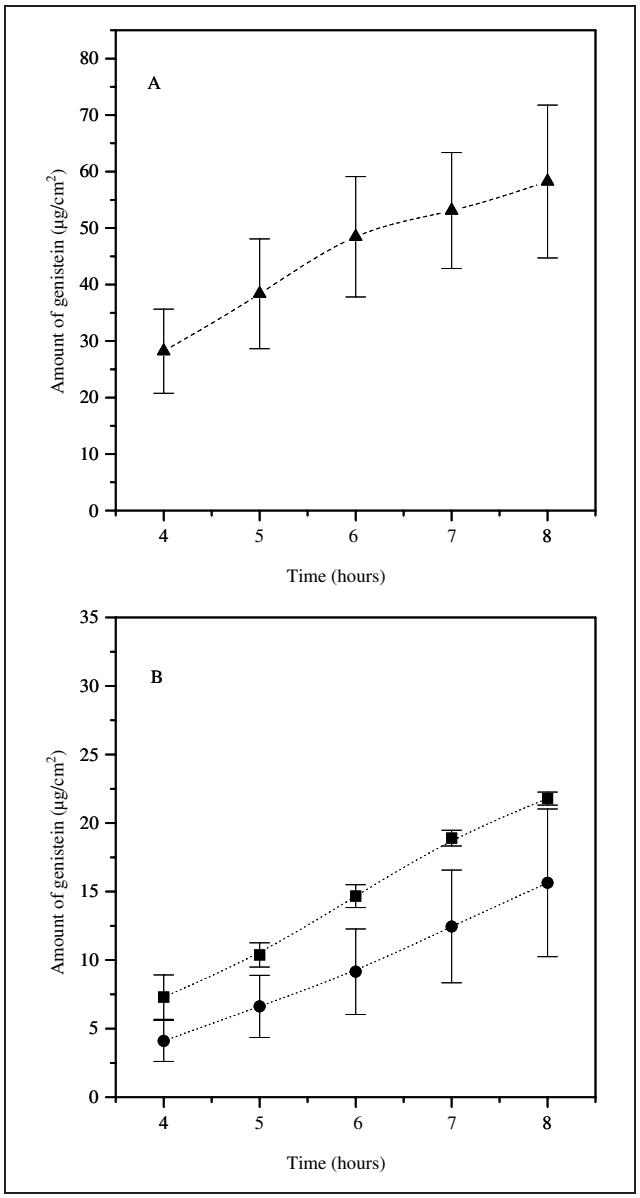

Fig. 3: Cumulative amount of genistein permeated through excised porcine skin. [A]: GEN intrinsic profile [B]: ODD-nanoemulsion (■) and MCT-nanoemulsion (●). The values represent the mean ± S.D. of three individual experiments

Table 3: Final composition of nanoemulsions (%, w/w)

	MCT	MCT/GEN	ODD	ODD/GEN
Genistein	–	0.10		0.10
Egg-lecithin	2.00	2.00	2.00	2.00
MCT	8.00	8.00	–	–
ODD	–	–	8.00	8.00
Water q.s.p.	100.00	100.00	100.00	100.00

MCT: medium chain triglycerides; ODD: Octyldodecanol; GEN: genistein

3. Experimental

3.1. Chemical and reagents

Egg-lecithin and medium chain triglycerides (caprylic/capric triglycerides) were kindly donated by Lipoid GmbH (Ludwigshafen, Germany). Octyldodecanol was obtained from Delaware (Porto Alegre, Brazil). Genistein was purchased from Sigma (São Paulo, Brazil). Ultrapure water was obtained from a Milli-Q apparatus (Millipore, Billerica, USA). Acetonitrile, methanol and ethanol LC grade were obtained from Merck (Darmstadt, Germany).

3.2. Preparation of nanoemulsions

Nanoemulsions containing genistein were prepared using a spontaneous emulsification procedure. Briefly, this method consists of injecting an organic phase containing components of the oil core into the water phase under magnetic stirring. Subsequently, the organic solvent (ethanol) was removed by evaporation under reduced pressure at 40–45 °C. Typically, two kinds of formulations composed by either medium chain triglycerides or octyldodecanol as oil core were prepared. Genistein was added to the ethanol phase in order to have a final concentration in nanoemulsions of 1 mg/mL. Blank formulations were prepared under the same conditions in the absence of genistein as control formulations. The final composition (%, w/w) of nanoemulsions obtained in the absence (MCT or ODD) or in the presence of genistein (MCT/GEN or ODD/GEN) were presented in Table 3.

3.3. Characterization of nanoemulsions

The mean droplet size and ζ-potential of the emulsions were determined by PCS and electrophoretic mobility, at 25 °C and at an angle of 90°, respectively (3000HS Zetasizer, Malvern Instruments, England). The samples were adequately diluted in water for size determinations or in 1 mM NaCl solution for ζ-potential measurements. The viscosity of the nanoemulsions was evaluated by capillary viscosimetry. Five milliliters of each nanoemulsion were poured into a filling tube and transferred to the capillary tube (viscosimeter constant, k = 0.0212) by gentle suction. The time was recorded, in seconds, for the liquid to flow from the upper to the lower mark in the capillary tube. Results are the mean ± standard deviation of three batches.

Morphologic examination of emulsions was performed by means of TEM. Nanoemulsions were diluted at 1 : 10 ratio obtaining an oil phase concentration equal to 1% (v/v). Specimens for TEM visualization were prepared by mixing samples with one droplet of 2% (w/v) uranyl acetate solution. The samples were then adsorbed to the 200 mesh formvar-coated copper grids, left to dry, and examined by TEM (JEM-1200 ExII, Jeol, Japan).

3.4. Determination of genistein association efficiency

The determination of genistein content in the nanoemulsions was carried out by using the conditions previously described by Silva et al. (2007). Nanoemulsion aliquots of 0.25 ml containing genistein were appropriately diluted in methanol, filtered and analyzed by liquid chromatography (LC). For association efficiency study, sample of nanoemulsions were added to ultrafiltration membranes (Ultrafree-MC 10,000 MW, Millipore) and centrifuged at 5,000 rpm. Free genistein was determined in a clear ultrafiltrate obtained by separation of the water phase. The association efficiency (%) was estimated by the difference between the total genistein content in nanoemulsions and free genistein concentration found in the ultrafiltrate.

3.5. Genistein solubility in oils

The solubility of genistein in oils was determined at room temperature. An excess of genistein was added to samples of either MCT or ODD. The mixtures were shaken for 24 h, centrifuged, and the supernatant was diluted in methanol for further analysis of soluble genistein by LC (Silva et al. 2007).

3.6. Differential scanning calorimetry (DSC)

DSC analysis was carried out in order to investigate the effect of egg-lecithin on the thermal profile of genistein. Samples of pure genistein, egg-lecithin and the mixture genistein/egg-lecithin were analyzed at the same ratio that was used in nanoemulsion formulations. The samples were sealed in an aluminum pans and scanned between 25–350 °C at a heating rate of 10 °C/min. using a Shimadzu DSC-60 (Shimadzu, Japan) under N_2 atmosphere. An empty aluminum pan was used as reference, and temperature was calibrated with indium (melting point 156.63 °C) and zinc (melting point 419.58 °C).

3.7. In vitro percutaneous permeation study

Percutaneous permeability of genistein was assessed using Franz type diffusion cells which presented a surface area for diffusion of 2.54 cm^2 and a receptor volume of 9.0 ml. Excised circular skins of pig ear were mounted between donor and receptor compartments having the inner part facing the inside part of the cell. Then, the skin was hydrated with phosphate buffer (pH 7.4) for 12 h at 37 °C. After that, the phosphate buffer in the receptor chamber was replaced by a methanolic solution (50%, v/v) in order to ensure sink conditions. The bathing solution was kept under controlled temperature (37 ± 1.0 °C) and stirring throughout the total time of experiment (8 h). An acetone solution (volatile reservoir) or nanoemulsions were placed in the donor compartment at a theoretical concentration of 1 mg genistein. Samples of 2.0 ml were withdrawn at hourly intervals and the same volume of fresh receptor fluid was added in order to keep a constant volume. The aliquots were diluted in methanol and analyzed by LC at 270 nm (Silva 2006). The results were expressed as mean ± standard errors of permeated genistein per unit surface area (μg/cm^2) as function of time (h) and a graph was plotted. Then, the steady-state flux was calculated from the slope of the resulting linear profile. The lag-time of diffusion corresponds to the intercept of the linear profile in the x-axis.

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