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Combined effect of liposomalization and addition of glycerol on the transdermal delivery of isosorbide 5-nitrate in rat skin

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Abstract

In this report, we investigated the combined effect of drug liposomalization and addition of glycerol on the transdermal delivery of isosorbide 5-nitrate (ISN) in rat abdominal skin *in vitro*. Occlusive application of both liposomal and aqueous ISN solution, with and without addition of 5% glycerol, showed that drug liposomalization and addition of glycerol has far-reaching implications for ISN permeation and accumulation in 4 and 8 weeks old rat abdominal skin. Using 8 weeks old rat abdominal skin, the optimal concentration of glycerol to be added to liposomal ISN was found to be 5%. The ISN mean values permeated through and accumulated in stripped 8 weeks old rat abdominal skin from those formulations described above were not significant different, which might indicate the combined effect of glycerol and liposomal ISN resides solely in the stratum corneum (SC). Based on previous reports, the enhancement effect of glycerol might be due to an increase in the SC hydration, and perhaps due to subtle changes in the lipid organization caused by penetration of liposomal lipids within the SC intercellular spaces. These data might provide evidence that glycerol action on SC is useful to facilitate skin permeation and accumulation of drugs formulated in liposome.
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Keywords: Skin penetration and accumulation; Liposomes; Glycerol; Isosorbide 5-nitrate; Transdermal delivery; Skin hydration

1. Introduction

The main barrier and rate-limiting step for diffusion of drugs across the skin is located in the outermost layer of the skin, the stratum corneum (SC), which contains flattened dead epidermal cells (corneocytes) embedded in hydrophobic lipid domains. The majority of these lipids form crystalline lamellar phases. Since the lipids regions in the SC form the only continuous structure, most drugs applied onto the skin permeate across the intercellular lipid lamellar regions (Bouwstra and Honeywell-Nguyen, 2002). This has been particularly illustrated by confocal studies (Simonetti et al., 1995), which revealed that fluorescent probes mainly diffuse along the intercellular tortuous pathway.

Besides a proper lipid organization, the level of hydration of the SC is one of the key elements for the skin barrier function. The level of skin hydration is a function of the humidity of the environment and the hygroscopic properties of the SC. Glycerol, also known as glycerin, is a polyol with three hydroxyl groups through which hydrogen bonds can be formed with water molecules, bringing about considerable water uptake (Batt and Fairhust, 1986; Cohen et al., 1993; Murray and Wickett, 1996; Summers et al., 1996). A recent report indicated that glycerol is a major determinant of SC water retention, and mechanical and biosynthetic functions (Hara and Verkman, 2003). Further investigations have also been made concerning the mechanism of barrier improvement after damage with sodium dodecyl sulfate or tape stripping and glycerol treatment (Fluhr et al., 1999; Zhai and Maibach, 2001). It was shown that not only the barrier regenerated faster under the influence of glycerol but also the effect persisted for an extraordinarily long time. It seemed that glycerol creates a stimulus for barrier repair while it improves the SC hydration (Fluhr et al., 1999).

Most part of the available methods for improving percutaneous delivery facilitates drug transport by perturbing the

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SC barrier function. The topical formulations are therefore expected to change the hydration levels of the SC and modify the mechanical properties of the skin in order to achieve a high drug permeation and/or accumulation effect. Several groups have shown that liposomes enhance the penetration of drugs into the skin or enhance the transdermal flux of drugs (Lasch and Wohlrab, 1986; Egbaria et al., 1991; Lauer et al., 1995; Kirjavainen et al., 1996; Verma et al., 2003). The structure of liposomes, which mimics the organized lipid structure of the SC, offers a suitable strategy for achieving an accurate vehiculization of a particular compound and for incorporating additional lipid content that modifies the water barrier function of the skin (Fresta and Puglisi, 1997; Coderch et al., 1999; Schmid and Korting, 1993).

In this regard, we postulated that due to the special properties of liposome and glycerol described above, association of a liposomal drug formulation with glycerol could improve the hydration of the skin and create a temporary environment appropriate for drug diffusion through the intercellular spaces to the deeper layers of the skin and posterior blood circulation. So that, in this report we investigated the combined effect of liposomalization and addition of glycerol on the transdermal delivery of isosorbide 5-nitrate (ISN), in rat abdominal skin *in vitro*. Occlusive application of the samples was used since the vast majority of studies investigating percutaneous penetration are conducted under this condition. Occlusive application leads to the entrapment of water which is expected to substantially change many properties of the skin, including hydration and permeability of the skin barrier to some exogenous chemicals (Treffel et al., 1992). Permeation and accumulation studies in stripped rat abdominal skin was realized in order to determine whether the combined effect of drug liposomalization and glycerol was restricted solely to the SC.

2. Materials and methods

2.1. Materials

Egg yolk phosphatidylcholine (EPC) was supplied by Nippon Oil & Fats (Tokyo, Japan). Cholesterol (CHOL), acetaminophen, chloroform and methanol were purchased from Wako Pure Chemical (Osaka, Japan). ISN was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). The other reagents were analytical grade and used as received.

2.2. Preparation of liposomal ISN and liposomal ISN containing 5% glycerol

Conventional liposomes were prepared as described previously (Barichello et al., 2006). Briefly, EPC (100 mg), ISN (20 mg) and CHOL (10 mg) were dissolved in an appropriated volume of a mixture of chloroform and methanol (1:1). The mixture was dried up to a thin drug-lipid film under vacuum using a rotary evaporator (Buchi, Flawil, Switzerland). This drug-lipid film was kept under high vacuum over night to remove the traces of chloroform. The dried drug-lipid film was hydrated with 5 ml of Milli-Q water to obtain a lipid suspension and the particles were downsized by applying sonication for 1 h at 4 °C using a 3 mm probe sonicator (Branson, CT, USA). The mean diameter of the liposomes was determined by a submicron particle size analyzer (NICOMP380, CA, USA). Liposome size presented two peaks at 50 nm (85%) and 170 nm (15%) (Woodbury et al., 2006). The amount of ISN encapsulated in liposomes was $9.19 \pm 0.43\%$. The liposome suspension was diluted with Milli-Q water to give a final ISN concentration of 1 mg/ml. For preparing the liposomal ISN containing glycerol, the required amount of glycerol was added to the liposomal suspension and the volume was adjusted with Milli-Q water to give a concentration of 1 mg/ml of ISN. The concentrations of glycerol evaluated were 2.5, 5.0, 10.0 and 20.0% (w/w). The liposome size after addition of the different conc. of glycerol did not vary from the initial liposome size.

2.3. ISN solution and ISN solution containing 5% glycerol

Ten milligrams of ISN was dissolved in an appropriate volume of Milli-Q water and the volume complete to give a final concentration of 1 mg/ml of ISN. For preparation of the ISN solution containing 5% glycerol, the required amount of glycerol was added to the ISN solution and the volume complete to give a final concentration of 1 mg/ml of ISN.

2.4. Determination of isosorbide 5-nitrate

The concentration of ISN was determined by a reverse phase high performance liquid chromatography method (HPLC). The samples were injected into a HPLC system composed of an auto sampler (Waters WISP M712, Milford, USA), a pump (Waters600E, Milford, USA), an ultraviolet detector (Waters 486 UV/VIS, Milford, USA), an integrator (C-R4A, Shimadzu Co., Kyoto, Japan) and a column (Capcell Pak C18 MG 5 μm 4.6 × 250 mm, The Shiseido Fine Chemicals, Tokyo, Japan). The mobile phase methanol and water (20:80) was eluted at a flow rate of 1.0 ml/min and the UV detector was set to 220 nm wavelength. The column temperature was 40 °C. At this condition, the retention time of ISN was around 11.5 min and the minimal amount detected was 0.025 μg/ml. Acetaminophen was used as an internal standard in the concentration 0.0002% (w/v).

2.5. In vitro drug permeation through rat skin

Freshly acquired abdominal skin from Wistar male rats (Japan SLC, Shizuoka, Japan) 4 or 8 weeks old was used. The whole abdominal region was carefully shaved and excised immediately before the experiment. The fat tissue was separated from the skin, not damaging the dermis. For the study using stripped skin, the SC was completely removed by stripping the skin at least 20 times with a mending tape. On the Franz diffusion cell, the skin sections were mounted with a nominal surface areas of 1.77 cm^2 and a receiver compartment of 10 ml capacity. The epidermal side of the skin was exposed to ambient environment while the dermal side was bathed in a phosphate buffer saline (PBS) (pH 7.4). The receptor fluid was mixed with a mag-

netic stirring bar at 400 rpm. PBS was kept at 37 ± 0.5 °C by a water jacket. All bubbles were carefully removed between the underside of the skin and the solution in the receiver compartment. One ml of each formulation containing ISN (1 mg/ml) was applied onto the skin surface area using a micropipette. Occlusive application was chosen due to its ability to maintain a drug concentration in the skin avoiding the gelation of conventional liposomes by complete dehydration of the formulation that may prevent partitioning of the drug into the deeper layers of the skin (Kim et al., 1998). The donor recipient was sealed with a laboratory film. At least six diffusion cells were used for each sample and every experiment was realized twice. One hundred microliters samples were collected from the receiver recipient at 4, 8, 12 and 24 h after the formulation were applied to skin, and sink conditions were kept by adding the PBS solution. After 24 h, the experiment was stopped and the diffusion setup was dismantled and the skin was removed to determine the ISN in skin as follows. ISN amount in samples was determined as described in the Section 2.4. The volume of sample injected was 20 μl.

2.6. In vitro drug accumulation in rat skin

The skins that were used in the permeation study were carefully washed with distilled water and their surfaces were dried up. In samples where intact skin was used, the SC was completely removed by stripping with a mending tape. The amount of drug in the epidermis and dermis was determined. Then, the skins were desiccated for about 3 h and weighed. The samples were put in a 5 ml sample vial and cut in small pieces. After that, one ml of methanol containing the internal standard (acetaminophen) was added and homogenized for about 30 s using a homogenizer (Ishii Seisakusho, Osaka, Japan). This operation was repeated 10 times and the collected homogenized skin was centrifuged at 10,000 × *g* for 10 min at 4 °C. The upper phase was taken up and filtered using an Ekicrodisc 0.45 μm filter unit (Gelman Sciences Japan, Tokyo, Japan). ISN amount in samples was determined as described in the Section 2.4. The volume of sample injected was 150 μl.

2.7. Statistical analysis

As the permeation and accumulation data were normally distributed (Kolmogorov–Smirnov test), a one-way analysis of variance (ANOVA) was used. Thereafter, post hoc multiple comparisons of data were analyzed with the Tukey–Kramer test. In all the tests, the level of significance was set at $P < 0.05$, and calculations were handled by the Graph Pad Stat View software (Abacus Concepts, Inc., CA, USA).

3. Results

3.1. ISN permeation and accumulation in rat skin

The permeation profiles of ISN through 8 weeks old rat abdominal skin from various formulations are shown in Fig. 1A. The amount of ISN permeated through rat abdominal skin from a liposomal formulation containing 5% glycerol was significantly higher compared to the ISN permeated amount from the other formulations ($P < 0.001$). No significant difference in the permeated ISN mean values among the other formulations tested was observed.

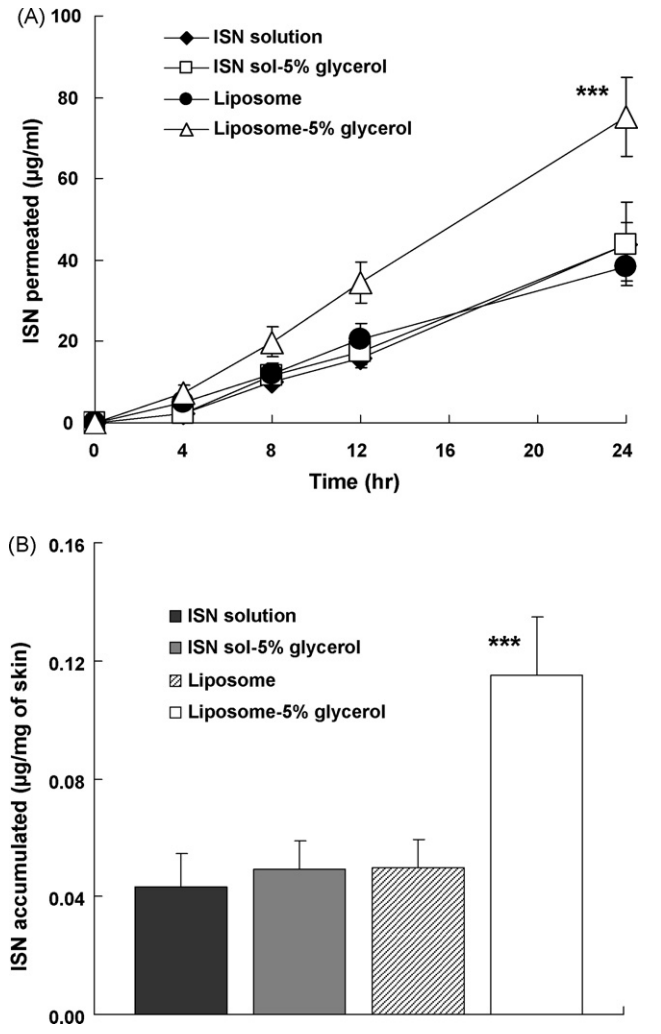

Fig. 1. Effect of various formulations on the cumulative amount of isosorbide 5-nitrate (ISN) permeated through (A) and accumulated in (B) 8 weeks old rat abdominal skin *in vitro*. Values represent the mean of six cells ± S.D. Statistics: significant difference in the ISN mean values permeated and accumulated at 24-h in 8 weeks rat abdominal skin from a liposomal ISN formulation containing 5% glycerol compared to other formulations (***$P < 0.001$).

The accumulation profiles of ISN in viable (dermis and epidermis) skin from various formulations are shown in Fig. 1B. The amount of ISN accumulated in the viable skin after a 24-h permeation study from a liposome formulation containing 5% glycerol was significantly higher compared to the accumulated ISN amount from the other formulations tested ($P < 0.001$). No significant difference in the accumulated ISN mean values was observed among the other formulations tested. These results clearly demonstrated that addition of glycerol to ISN-formulated in liposomes significantly improved drug permeation and accumulation in 8 weeks old rat abdominal skin.

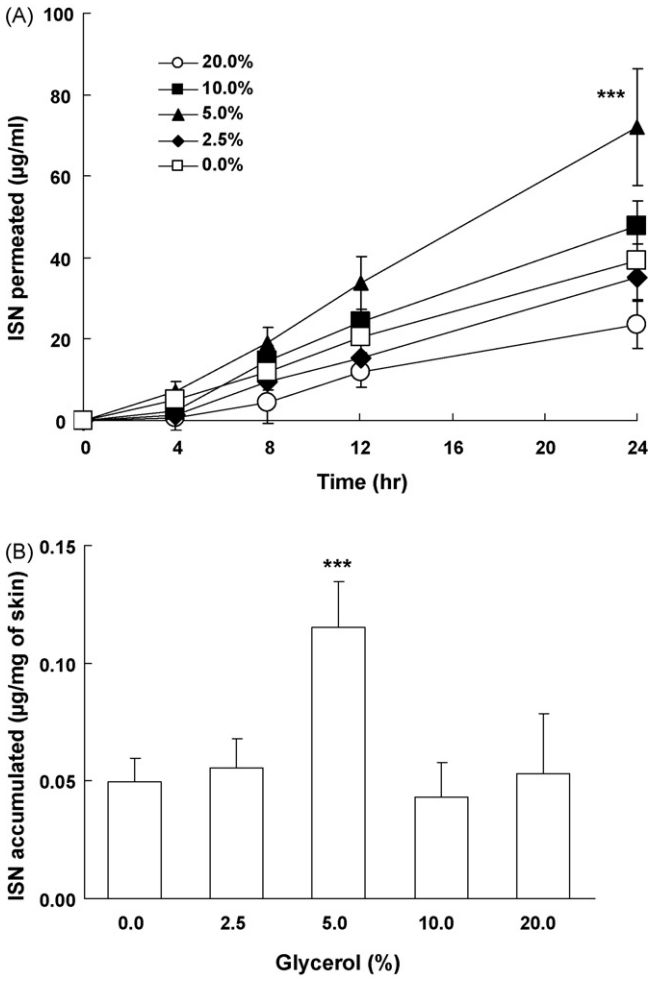

Fig. 2. Effect of glycerol concentration on the amount of isosorbide 5-nitrate (ISN) permeated through (A) and accumulated in (B) 8 weeks old rat abdominal skin from a liposome formulation *in vitro*. Values represent the mean of six cells ± S.D. Statistics: significant difference in the ISN mean values permeated and accumulated at 24-h in 8 weeks rat abdominal skin from a liposomal ISN formulation containing 5% glycerol compared to all other formulations (***$P < 0.001$).

3.2. Effect of glycerol concentration on skin permeation and accumulation of liposomal ISN

The effect of the glycerol concentrations (0, 2.5, 5, 10 and 20%) on ISN permeation and accumulation in 8 weeks old rat abdominal skin from a liposomal formulation are shown in Fig. 2A and B, respectively. It is observed that addition of 5% glycerol to the liposomal formulation permeated effectively more ISN through rat abdominal skin compared to the other glycerol concentrations evaluated ($P < 0.001$). It is also observed that addition of 10% glycerol to the liposome formulation permeated effectively more ISN through rat skin than addition of 20% glycerol ($P < 0.001$). The liposomal formulation without glycerol also permeated more ISN than addition of 20% glycerol ($P < 0.05$). Accordingly, the amount of ISN accumulated in the viable skin remarkably increased with addition of 5% glycerol to the liposome formulation compared to the other glycerol concentrations investigated ($P < 0.001$). No significant difference in the permeated ISN mean values among the other formulations tested was observed. It seems that addition of 5% glycerol leaded to the highest level of ISN permeation and accumulation in rat abdominal skin from a liposome formulation.

3.3. ISN permeation and accumulation in stripped rat skin

In order to clarify whether the effect of adding 5% glycerol to liposomal ISN was restricted to the SC, the permeation and accumulation study was carried out after completely stripping the 8 weeks old rat abdominal skins. The results of ISN permeation and accumulation in stripped skin are presented in Fig. 3A and B, respectively. No significant difference was observed among the permeated ISN amounts through stripped rat abdominal skin from the formulations evaluated. In the same way, the amount of ISN accumulated in stripped rat abdominal skin from the formulations evaluated was not significantly different. These findings may indicate that the permeation effect of glycerol on liposomal ISN resides solely in the SC.

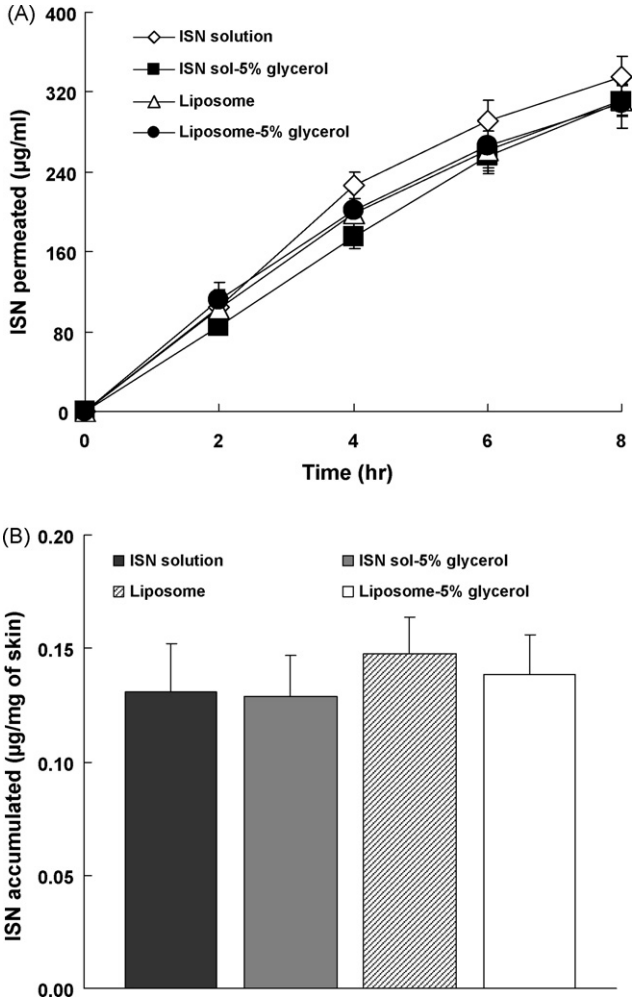

Fig. 3. Effect of various formulations on the cumulative amount of isosorbide 5-nitrate (ISN) permeated through (A) and accumulated in (B) stripped 8 weeks old rat abdominal skin *in vitro*. Values represent the mean of six cells ± S.D.

3.4. ISN permeation and accumulation in 4 weeks old rat skin

The permeation profiles of ISN through 4 weeks old rat abdominal skin from various formulations are shown in Fig. 4A. In 4 weeks old rat abdominal skin, the permeated ISN amount from the liposomal formulation containing 5% glycerol was significantly higher compared to the permeated ISN amount of an ISN solution ($P < 0.001$). No significant difference was observed in the permeated ISN mean values among the other formulations.

The accumulation profiles of ISN in 4 weeks old rat abdominal skin from various formulations are shown in Fig. 4B. The liposomal formulation containing 5% glycerol accumulated significantly more ISN in the viable skin after a 24-h permeation study compared to liposomal ISN and the aqueous ISN solution, with or without 5% glycerol ($P < 0.01$). No significant difference was found among the accumulated ISN mean values of the other formulations tested. These results demonstrated that addition of glycerol to the liposomal ISN did not improve drug permeation and accumulation in 4 weeks old as effective as in 8 weeks old rat abdominal skin.

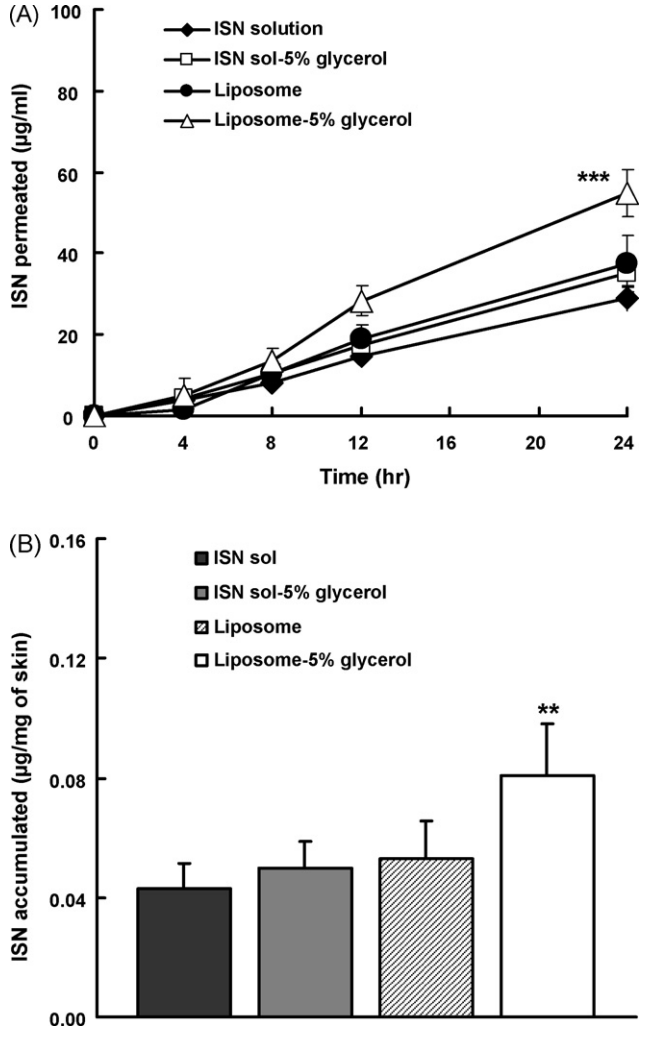

Fig. 4. Effect of various formulations on the cumulative amount of isosorbide 5-nitrate (ISN) permeated through (A) and accumulated in (B) 4 weeks old rat abdominal skin *in vitro*. Values represent the mean of six cells ± S.D. Statistics: (A) significant difference in the ISN mean values permeated through 4 weeks rat abdominal skin at 24-h from a liposomal ISN formulation containing 5% glycerol compared to other formulations (***$P < 0.001$) tested. (B) Significant difference in the ISN mean values accumulated in 4 weeks rat abdominal skin at 24-h from a liposomal ISN formulation containing 5% glycerol compared to other formulations (**$P < 0.01$).

4. Discussion

In this study, the combined effect of glycerol and formulation of ISN in liposome on its transdermal delivery in 4 and 8 weeks old rat abdominal skin was investigated. Our findings demonstrated that association of liposomal ISN with glycerol significantly improved the permeation and accumulation of ISN in 4 and 8 weeks old rat abdominal skin compare to an ISN aqueous solution, with and without glycerol, and a liposomal ISN formulation. In addition, the amount of ISN permeated through and accumulated in 8 weeks old rat abdominal skin was found to be higher than in 4 weeks old rat abdominal skin. Indeed, results clearly demonstrated that the effect of the liposomal ISN formulation containing 5% glycerol was restricted to the SC layer since without the SC barrier, all tested formulations could similarly increase the permeation and accumulation of ISN in the viable skin.

Studying the barrier function of SC, Friberq and Osborne (1985) has proposed that a liquid crystalline state of the intercellular lipids is required for optimum barrier function. The balance between liquid and solid crystalline phases of the lipids was influenced not only by hydration, but also by the proportion of unsaturated fatty acids and probably other unknown factors. *In vitro* experiments have shown that glycerol inhibits the transformation of lipids from liquid to solid crystalline state (Friberq and Osborne, 1985). As the structured lipids of the SC constitute the primary permeability barrier of the skin (Onken and Moyer, 1963; Elias, 1981; Grubauer et al., 1989), it follows that exogenous glycerol could work as a lipid phase-modulating molecule of lipids in the intercellular space of the SC, and contribute to improve the uptake of water to increase the hydration level in order that disruption of lipids ultrastructure could occur (Warner et al., 1999).

Glycerol is well known for its humectant and hygroscopic properties. A recent study has provided evidence for glycerol as a water-retaining humectant in the SC (Hara and Verkman, 2003). It was demonstrated that normal SC hydration requires endogenous glycerol (Fluhr et al., 2003) and that both circulatory and sebaceous gland origin glycerol, compromises a water-extractable pool that influences SC hydration in humans (Choi et al., 2005). Moreover, a recent work has shown that keratinocytes in the SC of human skin equivalents, a reconstructed SC, have the ability to swell when a sufficient amount of water is provided, and that larger water domains, however, are also present in the intercellular regions (Bouwstra et al., 2008). There is therefore a considerable role for externally applied modifiers of SC plasticity, such as glycerol, to promote an increase in skin hydration, particularly within the SC barrier, with consequent swelling of corneocytes.

Complementary, liquid-state vesicles were demonstrated to induce perturbations in lipid organization in deeper layers of the SC and formation of water pools within the SC (Hofland et al., 1995b; Van den Bergh et al., 2001). An investigation on the effect of the composition of liquid-state liposomes on SC lipids organization demonstrated that marked changes in the SC ultrastructure was induced by a formulation containing a high fraction of phosphatidylcholine (Hofland et al., 1995a). The corneocytes were considerably swollen as compared to untreated skin, and the ultrastructure of intercellular lipid lamellae showed flattened spherical structures, indicating that a substantial amount of liposome material was incorporated in the SC intercellular regions (Hofland et al., 1995a).

Considering that the composition of the liposomes used here has a high concentration of EPC, and on the basis of the glycerol effect on skin reported in literature, thus the possible effects of glycerol could be through its humectant and lipid solubilizing properties. The strong attractive forces are likely important for the self-assembly of amphiphilic molecules in water. On the other hand, reorganization to form an interface involves a significant cost, if the interface is remained. This cost or free energy of formation needs to be compensated for forces that favor separation of the system into different phases. Therefore, we speculated that the free energy, required to remove a molecule from well hydrogen bonded interior bulk water to the lesser hydrogen bonded surface, is dependent on the glycerol concentration. At the conc. of 5%, glycerol might facilitate the release of individual lipids and/or lipid fragments from liposome. The released lipids could eventually penetrate the SC intercellular regions and occasionally be incorporated into the lipid layer that composes the intercellular spaces. This could imply enough plasticity and hydration to the environment of the SC intercellular spaces to become even more attractive for hydrophilic moieties permeation. This effect might thus facilitate an uptake of water by the SC intercellular lipids which fact could enormously facilitate the permeation of a drug through the SC intercellular spaces (Warner et al., 2003; Hara and Verkman, 2003; Choi et al., 2005; Bouwstra et al., 2008). However, at lower concentration of glycerol, the effect of hydration is not enough to overcome the free energy of the system, while at higher concentration of glycerol, the solubilization of lipids could importantly compromise the overall physicochemical properties of the system. This thereafter may lead that other concentration of glycerol gave less permeation of ISN compared to 5% of glycerol, by altering the flux of lipids and drug through the skin.

In addition, recent studies have suggested that supplementing intercellular lipids of the SC in aging populations or in people with dry skin can stimulate the functioning of the skin. In particular, Coderch et al. (2002) has evaluated the protection of healthy skin against detergent induced dermatitis using two different SC lipid mixtures formulated as liposomes. Increase in water holding capacity is obtained only when the formulations applied are structured as liposomes. This was slightly more pronounced for aged skin. In agreement, the amount of ISN permeated through and accumulated in 8 weeks old rat skin (Table 1) was significantly higher compared to the permeated ISN mean value in 4 weeks old rat abdominal skin after a 24-h permeation study

Table 1
Comparison of the cumulative amount of isosorbide 5-nitrate (ISN) permeation and accumulation in 4 and 8 weeks old rat abdominal skin from various formulations after a 24-h permeation study *in vitro*

	4 weeks	8 weeks
Permeated ISN (μg/ml)	54.77 ± 5.80	75.26 ± 9.60**
Accumulated ISN (μg/mg of skin)	0.810 ± 0.017	1.149 ± 0.012*

Values represent the mean of six cells ± S.D. Statistics: significant difference in the ISN mean values permeated through (**$P < 0.01$) and accumulated in (*$P < 0.05$) 8 weeks compared to 4 weeks old rat abdominal skin from a liposomal ISN formulation containing 5% glycerol.

($P < 0.01$), suggesting that addition of 5% glycerol to a liposomal ISN formulation improves ISN permeation and accumulation in aged rat abdominal skin.

Since elder populations is increasing world-wide, these results support the possibility of achieving two beneficial effects: of delivering a drug across the skin by using compounds that are naturally found in the skin and, at the same time, supplementing the skin with essential compounds that stimulate the functioning and reconditioning of the skin, which, as a result of age, health and environment conditions are presented in skin at suboptimal levels, without disturbing the intercellular lipids and/or SC organization.

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