약제학회지(2007), 제 37 권 제 5 호
J. Kor. Pharm. Sci., Vol. 37, No. 5, 281-286 (2007)

Transdermal Delivery of Porcine Placenta Extracts using Linolenic Acid-based Emulsion Formulations

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(Received October 2, 2007 · Accepted October 17, 2007)

ABSTRACT – For transdermal delivery of porcine placenta extract (PPE), various emulsion formulations were prepared and evaluated. Polysorbate surfactants were used as emulsifiers and various C-18 unsaturated fatty acids as enhancers. The skin permeation of PPE was tested using a cellulose nitrate membrane-loaded Franz cell apparatus. Among emulsifiers, Tween 20 provided higher penetration effect than did Tween 80. Meanwhile, of various fatty acids, linolenic acid (18:3) revealed the highest skin permeation of PPE than the other C-18 unsaturated fatty acids. Stability of PPE emulsions was determined by cycles of freezing and thawing processes. The stability of emulsions depended on the percentage of Tween 20. Minimum 20% of Tween 20 was required to stabilize emulsions at room temperature for several days. Taken together, our results suggest that Tween 20 and linolenic acids might be key components to formulate PPE emulsion to provide the desirable skin permeability and stability.

Key words – Porcine placenta extract, Emulsion, Unsaturated fatty acid, Transdermal delivery

Placenta extracts have been used to cosmetic and pharmaceutical products for whitening effect and oxidative related diseases.[1] When it comes to the sources of placenta tissues, human placenta extracts have been most highly favored source of placenta extract. However, the use of human placenta extract is limited due to the ethical problems in collecting the human placenta tissues from human. Alternatively, sheep placenta extracts have been used to replace human placenta extracts. But, the use of sheep placenta extracts carries the risk of spongiform encephalophy.[2] To avoid these problems, porcine placenta extracts (PPE) are emerging as a new industrial source of placenta. PPE is regarded as a suitable alternative of human placenta extract due to genetically high homogeneity between human and porcine placenta.[3,4]

PPE-containing cosmetic materials have been advertised to provide good skin permeability, and exert pharmacological effects such as skin whitening and anti-aging.[5] However, there has been little evidence-based approach to test whether the major components of PPE can be permeated through cosmetic formulations, and if so whether there exists key components to enhance the skin permeation of PPE.

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In this study, we formulated PPE in various emulsion compositions containing different emulsifiers and fatty acid enhancers. Here, we report the effective components for the improved skin permeation of PPE using W/O/W emulsion formulations.

Materials and methods

Materials

PPE was provided from Dr. Jeong Ho Kim at Seogang University (Seoul, Korea). Oleic acid was purchased from Duksan Chemical Co. (Gyeonggi-do, Korea). Conjugated linoleic acid (CLA) was from Lipogen (Gyeonggi-do, Korea). Linoleic acid was from MP Biomedical (Solon, OH, USA). Linolenic acid was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Tween 20 was from USB (Cleveland, OH, USA). Tween 80 was from Sigma Chemical Co. (St. Louis, MO, USA). Isopropyl myristate was from Fluka (St. Louis, MO, USA). Spectra/Por®7 regenerated cellulose membranes were from Spectrum laboratories, Inc. (Laguna Hills, CA, USA).

Quantification of PPE using primary amine assay

Since the major components of PPE are amino acids, TNBSA (2,4,6-Trinitrobenzen Sulfonic Acid) method was employed to measure the levels of primary amine in PPE. An aliquot (50 μL) of samples were added to 300 μL of 0.01%

Table I–*The Formulations of Various PPE Emulsions (v/v)*

Emulsions	Porcine placenta extract (%)	Emulsifier (%)	Oil phase (%)	Water phase (%)
A	14	Tween20 (7)	Linolenic Acid (29)	50
B	13	Tween20 (14)	Linolenic Acid (27)	46
C	13	Tween 20 (20)	Linolenic Acid (25)	42
D	12	Tween 20 (24)	Linolenic Acid (24)	40
E	11	Tween 20 (28)	Linolenic Acid (22)	39
F	10	Tween 20 (32)	Linolenic Acid (21)	37
G	13	Tween 20 (20)	Oleic Acid (25)	42
H	13	Tween 20 (20)	CLA (25)	42
I	13	Tween 20 (20)	Linoleic Acid (25)	42
J	13	Tween 20 (20)	Linolenic Acid (25)	42
K	13	Tween 80 (20)	Linolenic Acid (25)	42
L	13	Span 85 (20)	Linolenic Acid (25)	42

TNBSA solution. The samples were mixed while shaking at 300 rpm at 37°C for 2 hours. The optical density values were measured at 450 nm using a microplate reader. The calibration curves of primary amine concentrations were established using various concentrations of L-lysine solutions.

Preparation of multiple emulsions

Primary emulsions were prepared with various C-18 unsaturated fatty acids. Before making primary emulsions, PPE (100 μL) and emulsifiers (150 μL) were vigorously homogenized. Fatty acids (200 μL) were then added to the mixture (350 μL) of PPE and emulsifier, and homogenized vigorously at room temperature for 1 minute. Phosphate buffered saline (PBS) was added to the primary emulsions and homogenized for 1 minute to provide multiple W/O/W emulsions. As oil phase components, various carbon-18 unsaturated fatty acids were used. The fatty acids tested in this study include oleic acid (18:1), CLA (18:2), linoleic acid (18:2) and linolenic acid (18:3). To stabilize multiple emulsions, three emulsifiers such as Tween 20, Tween 80 and Span 85 were used. Table I shows the emulsifiers and oil phases used for the formulation of multiple emulsions.

In vitro skin permeability study

Modified Franz Cell Apparatus was used to test the skin permeability of PPE applied in various emulsion formulations. The diameter of Franz cell apparatus was 15 mm, and a diffusion area was 1.76 cm^2. Moreover, the receptor volume was 10 mL. Spectra/Por®7 regenerated cellulose membranes (thickness of 65 μm and a molecular weight cut-off of 1000) were saturated by isopropyl myristate (IPM) at 37°C during 12 hours. Receptor chambers were filled with 10 mL PBS, and maintained at 37°C.[6] Coated magnetic bar was stirred at 700 rpm in the receptor medium during experiments. An aliquot (200 μL) sample was collected from the receptor phase at 0.5, 1, 3, 6, 12, and 24 hours after the application of PPE emulsions and replaced with the same volume of PBS immediately. A cumulative amount of PPE in the receptor medium was determined by measuring the concentration of amino acids with TNBSA method.

Stability test

The stability of PPE emulsion was tested by cycles of freezing and thawing methods. Various formulations of Tween 20-based PPE emulsions were prepared using the same volume of linolenic acid. PPE emulsions were frozen at −20°C and thawed at 60°C for 2 hours. The freezing-thawing cycles were repeated several times. The stability of emulsions was evaluated by the number of cycles which resulted in the visible separation between oil and water phases.

Results and discussions

Quantification of PPE

As a marker molecule to measure the skin permeation of PPE, the primary amine molecules were tested in this study. Although there may exist various types of compounds in PPE, the amino acids are considered as the major components of PPE.[7] Due to the portion of amino acids in PPE, currently the total nitrogen contents are used for quality control of PPE products. To measure the skin permeation of PPE major components, amino acids, we employed the primary amino acid-detection method. TNBSA calibration curve (Fig. 1) shows that there is a high correlation between the concentration of

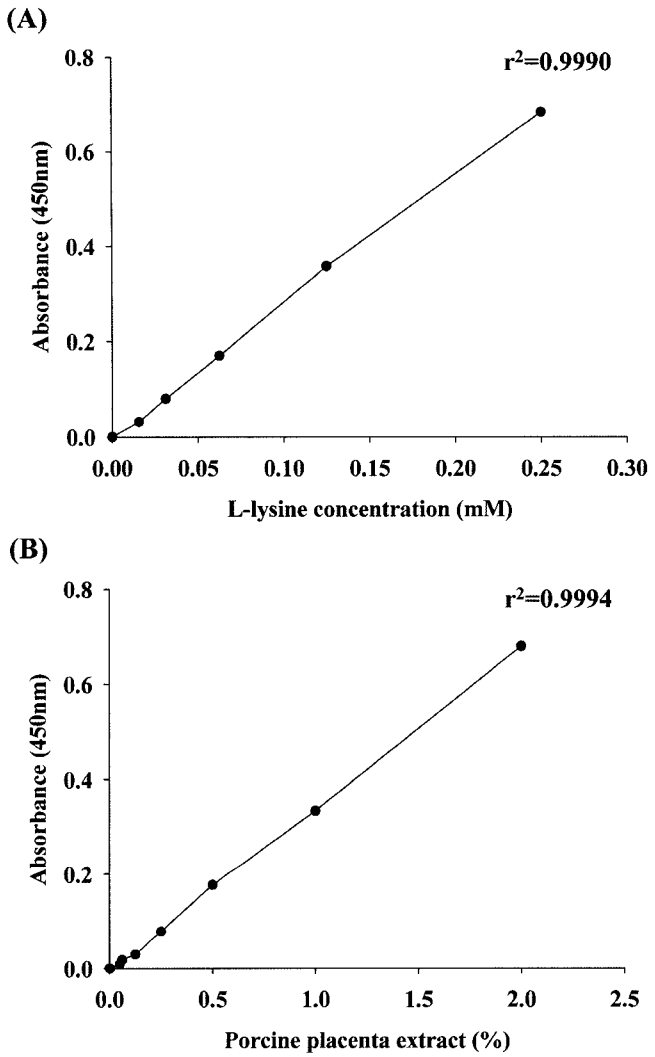

Figure 1–TNBSA calibration curve.
The representative TNBSA calibration curves are presented for (A) L-lysine concentration (mM) and (B) Porcine placenta extract concentration (%).

primary amino groups and the absorbance at 450 nm. TNBSA methods have been used to measure the concentrations of aminoglycosides and carboxylic acid to make amides.[8]

Compositions of multiple W/O/W emulsions of PPE

Multiple W/O/W emulsions were formulated using different types of emulsifiers and fatty acids.[9] The compositions of various multiple emulsions are described in Table I. As an emulsifier, Tween 20 was used. All the fatty acids were of the same carbon number, C-18, but different in double bond numbers. Linolenic, linoleic, conjugated linoleic, oleic acids were used for oil phase. These fatty acids were used as components of oil phase because fatty acids have been known to exert enhancer effects for transdermal delivery of various drug compounds.[10]

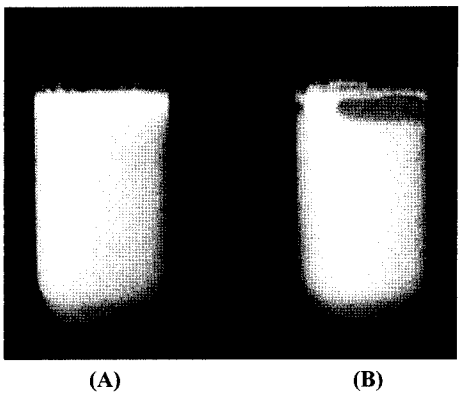

Figure 2–Evaluation of W/O/W multiple emulsion formulations. To evaluate the formulation of W/O/W emulsions, the emulsion was added with drops of hydrophilic sulfo-rhodamine in PBS(A) or with lipophilic sudan III in linolenic acid (B).

In particular, conjugated linoleic acid has been reported to modulate the lipid metabolism pathway.[11] To confirm the formulation of W/O/W emulsion, hydrophilic sulfo-rhodamine dissolved in PBS or lipophilic sudan III in linolenic acid was dropped into the emulsion (Figure 2). Sulfo-rhodamine diffused throughout the emulsion and formed homogenous pink colored emulsions (Fig. 2A). In contrast, Sudan III did not mix with the emulsion, and formed a separate layer on top of the emulsion (Fig. 2B). The homogenous diffusion of hydrophilic sulfo-rhodamine dye indicates that the emulsions formed in this study were W/O/W type.

Effects of emulsifier in the skin permeation of PPE

The type of emulsifiers affected the formation of multiple emulsions of PPE, and the skin permeation. In other studies, polysorbate surfactants were used for emulsifier because of good stability and relative non-toxicity.[12] Of polysorbate surfactants Tween 20 and Tween 80 were frequently used for cosmetic products and transdermal drug delivery system like emulsions.[13] Thus, in this study, we tested Tween 20, Tween 80, and Span 85 as emulsifiers for the stable formulation of multiple emulsions (Table I). However, Span 85 was not capable of forming stable multiple emulsions. Although Span 85-based emulsions showed higher levels of skin-permeated amino acids than did other emulsifiers until 12 hours in Franz cell study, the phase separation happened within a day after formation of emulsions (data not shown).

Due to the low stability of Span 85-based emulsions, we focused on the use of Tween 20 and Tween 80 as emulsifiers. Tween 20 showed higher skin permeation of PPE than did Tween 80 (Fig. 3). After application of PPE in 20% of Tween 20-based emulsions, the molarity of amino acids in the receptor phase was 0.0287 mM ± 0.0040 after 24 hours of incu-

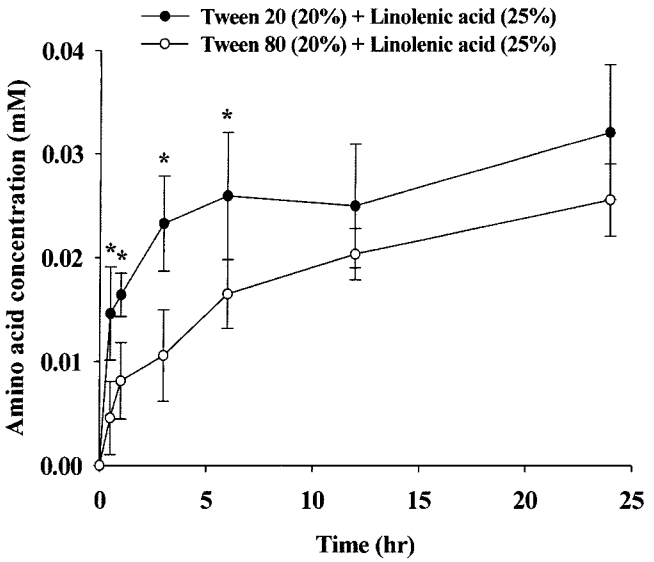

Figure 3—Skin permeation of PPE-derived amino acids formulated in multiple emulsions of different emulsifiers.
The skin permeation was tested using a Franz apparatus. The W/O/W emulsions of PPE were composed of linolenic acid and emulsifiers (n=4). Tween 20 or Tween 80 was used as an emulsifier.
*:$p<0.05$, Tween 20-based PPE emulsion significantly different from the other emulsion.

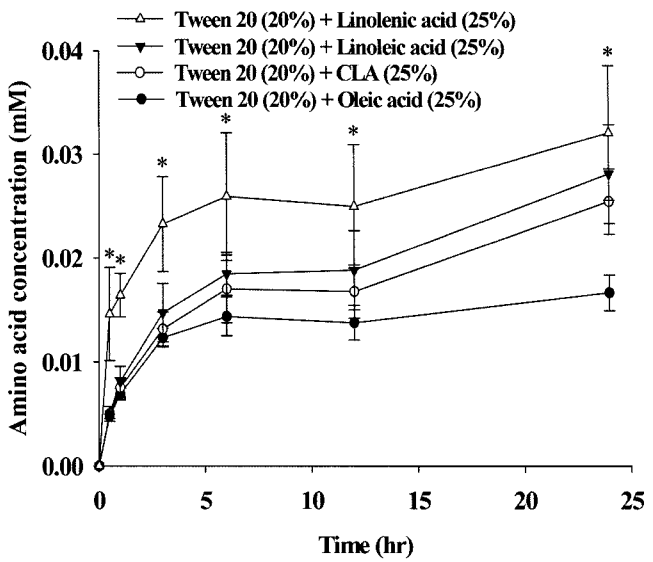

Figure 4—Skin permeation of PPE-derived amino acids formulated in various emulsions.
The skin permeation was tested using a Franz apparatus. The W/O/W emulsions of PPE were composed of Tween 20 and various C-18 fatty acids (n=4).
*:$p<0.05$, Linolenic acid-based PPE emulsions significantly different from the other emulsions.

bation. Tween 80 contains longer hydrocarbon chain (C18) than Tween 20 (C12). Hydrocarbon chains are involved in the hydrophobic interaction of the surfactant with the oil phase of the emulsions. Surfactants can affect oil-water interfacial tension and the particle size. Since the hydrocarbon chain size of Tween 80 is higher than that of Tween 20, there exist possibility that Tween 80-based emulsions may have been formed in larger sizes, limiting the skin permeation of PPE components.[14]

In this study, we used isopropyl myristate-soaked cellulose membrane as a model of artificial membrane. Initially, the skin of hairless mice was used to evaluate the permeation of PPE. However, due to the gradual release of amine-containing compounds from the skin, there were substantial time-dependent increases of TNBSA-positive signals in the permeation studies. To alleviate the artifacts, we used the isopropyl myristate-soaked cellulose membrane as an alternative substitute of mouse skins.

Effects of fatty acids in the skin permeation of PPE

Fatty acids used as oil phase components of multiple emulsions affected the skin permeation of PPE. Since Tween 20 provided the highest skin permeation, we used Tween 20 as an emulsifier to evaluate the enhancing effects of various fatty acids. All the fatty acids were of the same carbon number, C18, but different in the number of double bonds. Of various C-18 fatty acids with different double bonds, linolenic acid revealed the highest skin permeation of PPE (Fig. 4). Emulsions with linolenic acid provided two-fold higher levels of amino acids in acceptor solutions than did oleic acid-based emulsions. Especially, linolenic acid revealed three times higher initial skin permeation of PPE than the other C-18 fatty acids. Except linolenic acid, there was no significant difference among others fatty acids until 3 hours. The permeation enhancing activity of linolenic acid was followed by linoleic acid and CLA. Linoleic acid and CLA were similar in the number of double bonds, but different in the location of double bonds. Double bond of linoleic acid was located at C(9), C(12) while CLA was conjugated at C(9) and C(11). Oleic acid showed the lowest level of skin permeation of PPE.

Our results indicate that the number of double bonds in the fatty acids might be important for formulation of PPE emulsions with PPE. Fatty acids with more double bond showed the higher level of skin penetration. Previously, the skin permeation of arginine vasopressin was shown to be affected by fatty acids.[15] Although we observed significant differences in the skin permeation of PPE-derived amino acids by fatty acids of different double bonds, it may not be extended to the skin permeation of other chemical drugs. In case of indomethacin, other groups reported that the skin permeation of indomethacin was not affected by the differences of position and numbers of double bonds of unsaturated fatty acids (C18 chain).[16]

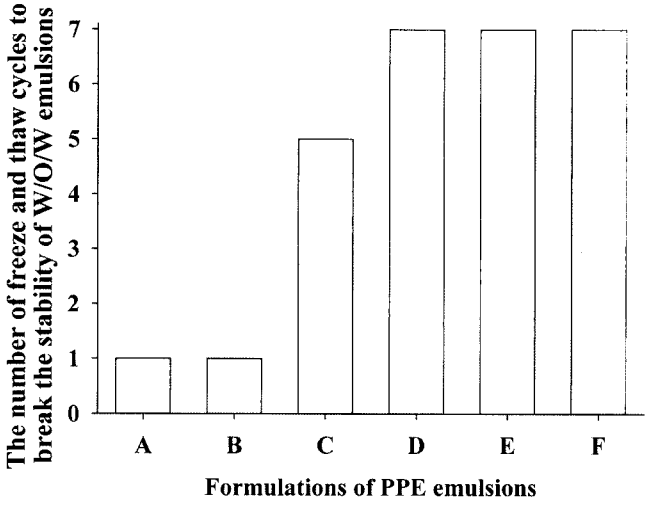

Figure 5–Physical stability of W/O/W emulsions.
All PPE emulsions were prepared as shown in table I (n=4). Emulsions were frozen at −20°C and thawed for 2 hours at 60°C. The number of freeze and thaw cycles which can break the W/O/W emulsions into two separate phases were recorded for each formulation (n=4).

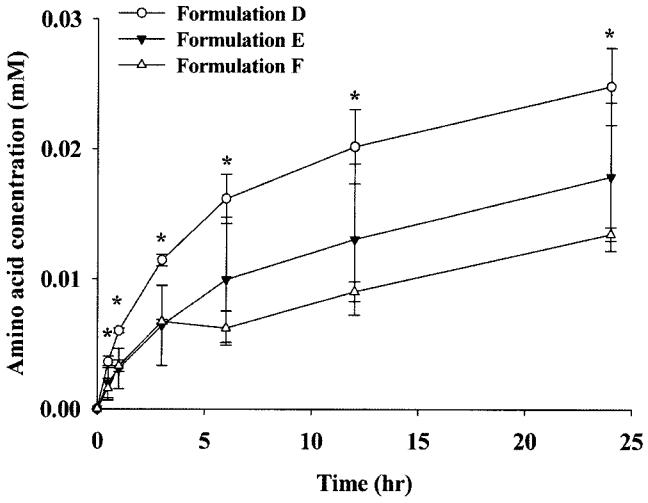

Figure 6–Skin permeation of PPE-derived amino acids formulated in linolenic acid-based emulsions.
All formulations are linolenic acid-based emulsions. Three formulations differ in the volume ratios of Tween 20 and linolenic acids.
*:$p<0.05$, Formulation D significantly different from formulation F.

Stability test

The stability of PPE formulations was evaluated by the number of freezing and thawing cycles. Among various formulations, Tween 20-based emulsion formulations were tested for stability since Tween 20 showed the higher skin permeation than did Tween 80. In the absence of Tween 20, the oil phase of multiple emulsion was immediately separated from the aqueous phase within few minutes after homogenation. Even though 7% and 14% Tween 20 emulsions were stable at room temperature for several days, these were broken after one week. When the concentrations of Tween 20 in the emulsions were lower than 20%, there was phase separation after only one cycle of freezing and thawing. As the concentrations of Tween 20 increased, the stability of emulsions also increased. Even though 7%, 14%, and 20% of Tween 20 based PPE emulsion was broken after 5 cycles. The other PPE emulsion based from 24% to 32% of Tween 20 was stable during freeze-thaw process until 7 cycles (Fig. 5).

When the W/O/W emulsions met cold air, oil in emulsions went to frozen. Water droplets in the oil were released during thawing of the oil.[17] These small drops increased in size and coalesced into large drops, leading to phase separation and break of the emulsion. Although we only measured the physical stability of the multiple emulsions using freeze and thaw method in this study, previous reports suggested that the chemical stability of active ingredients can be improved by W/O/W emulsions. The stability of ascorbic acid against oxidative degradation was shown to be enhanced in W/O/W multiple emulsion as compared to W/O or O/W primary emulsions. Moreover, the stability of thioglycolate in the inner aqueous phase in multiple emulsions was reported to be higher than those in primary emulsions.[18]

Effects of compositions in the skin permeation of linolenic acid-based PPE emulsions

Given the stability data, we further optimized the linolenic acid-based PPE emulsions by varying the ratios of linolenic acid and Tween 20. Formulations D, E, and F were chosen since they showed higher stability than other formulations tested in this study. Formulations D, E, and F were similar in that they all contained linolenic acid and Tween 20, but different in the ratios of two components (Table I). Among three formulations, formulation D showed significantly higher skin permeation of PPE-derived amino acids than did formulation F (Fig. 6).

Conclusion

In this study, multiple W/O/W emulsions were prepared for enhanced skin permeation of PPE major components. We found that the linoleic acid, the fatty acid with highest number of double bonds, in the emulsion formulations provided increased levels of PPE skin permeability, implying that the number of double bonds of fatty acid could affect the skin permeation of PPE in emulsion. Moreover, the type of emulsifier affected the skin permeation of PPE. Tween 20-based PPE

emulsion revealed higher skin permeation than did Tween 80-based emulsions. PPE emulsion equipped with Tween 20 as emulsifier and linolenic acid for oil phase may be a suitable combination for skin delivery of PPE using emulsion formulations. Given that PPE becomes gradually popular in the cosmeceutical field, our observation on multiple emulsion formulation studies may provide useful insights for further development of PPE products.

Acknowledgements

This study was financially supported by grants from Rural Development Administration BioGreen 21 program (20070501-034-001-007-03-00), from the Basic Research Program of Korea Science and Engineering Foundation (KOSEF R01-2007-000-20475-0), and from the Ministry of Science and Technology (F104AA010002-06A0101-00210), Republic of Korea.

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