Journal of Dermatological Science (2007) **45**, 135—137

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Journal of Dermatological SCIENCE

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LETTER TO THE EDITOR

Effect of ethosomal minoxidil on dermal delivery and hair cycle of C57BL/6 mice

Topical delivery of drugs from liposomal formulations has evoked a considerable interest for the last two decades. Many reports on enhancing percutaneous delivery are focused on the use of liposomes, because which can transport both hydrophilic and lipophilic compounds [1]. However, classic liposomes are of little or no value as carriers for transdermal drug delivery, because they do not easily penetrate the skin, but rather stay in the upper layer of the stratum corneum. Touitou et al. [2] have discovered that ethosomes, lipid vesicular systems with relatively high concentrations of ethanol, are very efficient in enhancing the skin permeation of a number of drugs. Their findings are supported by others' recent research results [3—5]. As a serial study, we used the well-defined C57BL/6 mouse model for hair research [6].

At first, phospholipon and minoxidil were dissolved in pure ethanol. Then the solution was slowly added into double distilled water in a fine stream and the mixture was mixed constantly at 1000 rpm for 30 min with a homogenizer. All the above procedures were performed in a sealed container full of nitrogen gas. The resulting ethosomal system components were extruded through a 0.2 μM diameter filter. The ethosomal calcein was also separated from the non-entrapped calcein by gel filtration on a Sepharose 4B column eluted with distilled water to form ethosome-entrapped beneficial calcein. The positive control was minoxidil group, in which naked minoxidil was dissolved in ethanol, propylene glycol, and water (60:20:20). The negative control was empty ethosome group without minoxidil.

Ethosome vesicles were examined by negative staining using a HITACHI H-600 electron microscope (TEM, Hitachi Ltd., Tokyo, Japan). Ethosomes prepared from 5% PC, 30% ethanol and water are multilamellar vesicles. The lamellae of ethosome vesicles were evenly spaced to the core. The size distribution of vesicles was determined by dynamic light scattering (DLS) using a computerized inspection system (Inc. Santa Barbara, CA, USA). The mean diameter of the vesicles was 194.7 nm. Drugs were quantitated by reverse-phase HPLC using a LC10Avp (Shimadzu, Tokyo, Japan). For minoxidil, the entrapment capacity of ethosomal vesicles determined by ultracentrifugation was $90 \pm 6\%$.

Four-week-old pre-shaved C57BL/6 mice were used for topical ethosome delivery of fluorescence and minoxidil to hair follicles in vivo. The ethosome entrapped with calcein or PE-rhodamine was directly applied on the mouse dorsal skin. Twenty-four hours after the first application, skin samples were obtained by punch biopsy for analysis.

Fig. 1 showed the confocal microphotographs of various formulations applied onto the mice skin under the same experimental conditions. Fluorescence intensity of the ethosomes formulations was high in the stratum corneum layer and in alive epidermis. Fluorescence was also observed in subepidermis. The above results suggested that ethosomes helped drugs reach deeper skin structures, such as pilosebaceous follicles. When the minoxidil was entrapped in the ethosome, 21.94 $\mu g/cm^2$ minoxidil was delivered into the skin after 12 h. However, less than 8.43 $\mu g/cm^2$ minoxidil was delivered in the control group where minoxidil is naked.

To synchronize the hair cycle, depilation of the dorsal skin was performed under anesthesia as previously described [6]. Four weeks after the synchronization of the hair cycle by depilation, all hair follicles in the depilated skin areas entered the telogen, based on the homogenous pink color of the dorsal skin. From this time point, the dorsal skin of each mouse was shaved under anesthesia using animal clippers. The mice were numbered by their weights, and were randomly divided into 3 groups (12 mice per group). Each group was applied different preparation respectively. The results showed that the time length needed for skin color changing from pink to black is 17.8 days ($P < 0.01$) and 9.3 days ($P < 0.01$) shorter in the ethosomal minoxidil group than that of blank ethosome group and naked minoxidil group, respectively. After

0923-1811/$30.00
doi:10.1016/j.jdermsci.2006.09.007

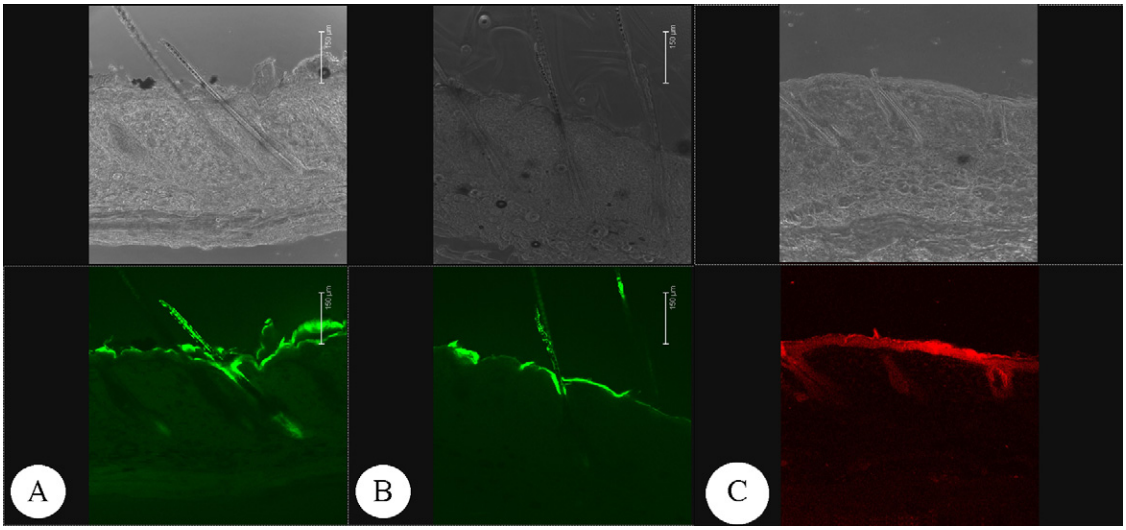

Fig. 1 Deeper permeation of the fluorescence in the calcein ethosomes group (A) than that in the naked calcein group (B). Dominant high accumulation of PE-rhodamine was observed around the hair follicles (C). CSLM. Magnification: 20×.

medicine application for 18 days, all of the hairs of ethosomal minoxidil treated group grew out of the mice skin. Histologically, these hair follicles became bigger, and the hair papillae were completely surrounded by the hair bulbs. In the hair bulbs, there were lots of newly formed melanocytes located in the deeper layer of the hypodermis. In addition, newly formed hairs and internal root sheathes were found in most hair bulbs. However, in the control group, the dorsal skins of mice were pink. There were fewer follicles, and less melanocyte formation. The termination of the hair shafts looked like a drumstick. The hair papillae were smaller, and they moved up to the arrectores pilorum attachment level. Representative pictures were shown in Fig. 2.

Neither erythoma nor edema was observed in the trial area of the ethosomal minoxidil group and the blank ethosome group, and the score of each group was lower than 0.49.

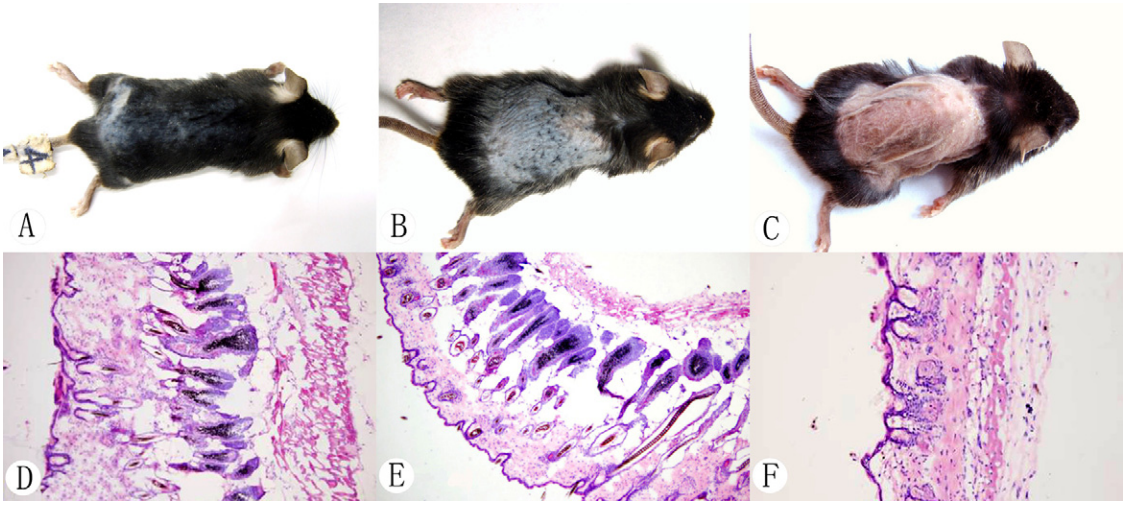

Fig. 2 At day18, hairs grew out of the skin in the ethosomal minoxidil treatment group (A). Hairs did not grow out of the skin surface in the naked minoxidil treatment group, although the color of the skin became black (B). The dorsal skins of mice were pink in the blank group (C). (D) Showed that in the ethosomal minoxidil treated group, hair follicles became bigger, and the hair papillae were completely surrounded by the hair bulbs. Inside the bulbs there were lots of newly formed melanocytes located in the deeper layer of the hypodermis. In addition, newly formed hairs and internal root sheathes could also be seen in most of the bulbs. The histological changes of the naked minoxidil treated group were similar to those of the ethosomal minoxidil treated group (E). In contrast, (F) showed that in the blank group, there were fewer follicles, fewer melanocytes, and smaller hair papilla. The termination of the hair shaft looked like a drumstick, and the papilla moved up to the arrectores pilorum attachment level. HE 200×.

Data presented here indicated that vesicular systems made of phospholipid, water and relatively high concentrations of ethanol were capable of enhancing delivery properties. Using the fluorescent probes of PE-rhodamine and calcein, we were able to visualize the penetration of lipophilic and hydrophilic molecules into the skin by ethosomal carriers. These qualitative results were supported by the delivery of minoxidil to the skin. The enhancement properties of the ethosomal carrier were also reported with cationic molecules such as propranolol and trihexyphenidyl [7].

Characteristics of the anagen include thickened dermal and epidermal layers of the skin, increased size of hair follicles, extension of follicles deep into the dermal adipose tissue, and initiation of melanin synthesis [8]. In the skin of C57BL/6 mice, melanogenesis is coupled to the anagen of hair cycle [8]. In our animal studies, topical minoxidil shortens the telogen of hair cycle, and causes resting hair follicles entering into the anagen prematurely. Minoxidil probably has a similar function in human. In addition, minoxidil may also prolong the anagen and increase the sizes of hair follicles [9]. As Messenger et al. [10] indicated that minoxidil probably has a similar function in hair growth for human. Based on the above animal tests, this observation suggests that ethosomal minoxidil may be a novel strategy for the management of hair growth disorders.

Acknowledgements

We are grateful to the Electron Microscopy Service from Key Laboratory of Environment and Genes Related to Diseases of Ministry of Education, School of Medicine, Xi'an Jiaotong University, Xi'an, Shaanxi, PR China for providing the CLSM and TEM equipment. We also would like to thank to Mr. Wang jiu-cheng and DR. Fu jing-guo of LiBang Enterprises, Xi'an, PR China for their HPLC technical assistance.

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17 May 2006