In Vivo Evaluation of Multi Lamellar Vesicle Liposome's Percutaneous Absorption and Stability

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Summary

We had prepared MLV liposome with Hibiscus Esculentus Ext.(HEE) which have fluorescent light in order to evaluate its percutaneous absorption about hairless rat skin. Then we investigated particle size of MLV using confocal laser scanning microscope(CLSM) and transmission electron microscope(TEM), respectively. Stability of MLV liposome and penetration of MLV liposome to hairless rat skin was measured by CLSM. As a result of experiments, MLV was globular shape and the range of particle size was 0.3-0.5μm mostly. Cream-type MLV had high stability comparatively. When we treated with MLV to rat skin, skin penetration was enhanced, especially, the optimum concentration of MLV on penetration to rat skin was 10%. Optimum penetration time was 6hr-12hr. And MLV-type HEE was more effective on percutaneous absorption than HEE-cream or liposome-type HEE.

I. Introduction

The human skin constitutes a vast physical barrier, protecting the body from myriad mechanical, chemical damages and microorganisms. Skin also controls a vasculature, sweating and percutaneous absorption system. In general, skin comprises a stratified epithelium on the outer layer and successively epidermis, dermis and hypodermis. The epidermis and, especially a stratified epithelium control minerals, salts and water between the organism and its environment. The dermis, if the epidermis were removed by stripping the skin, would have a complete permeability(1).

In theory, percutaneous absorption has two pathways(2). One is transmission through the epidermis and the other is penetration through sweet's pore. It has been studied for compounds, vehicles, application time and vasoconstrictors in enhancing percutaneous absorption, and liposome was developed as a representative vehicle for penetration enhancer(3).

In general, liposome, as a local delivery system, has been investigated for enhancing percutaneous absorption of steroid drug, particularly local depots and leading to decrease

an undesirable side effect of systemic absorption(4). Especially, multilamellar vesicle(MLV) liposome can contain not only water-soluble compound but also fat-soluble compound in lipid bilayer. Also, MLV's size and shape can be easily changed, and MLV consist of natural phospholipid has no toxicity in living body(5).

In the study of MLV penetration of skin, pig(6), rhesus monkey(7) and hairless rat(8) having a similar human skin were used. Particularly, hairless rat has the advantage of avoidable depilation which can affect skin barrier function(9).

Meanwhile, skin is composed of blood, gland, nerve, connective tissue and fats and so on. The in vitro system cannot maintain living skin condition during the measurement of MLV penetration of skin, therefore the in vivo system is suitable for the determining MLV penetration of skin(10).

The most common available in vitro system, among various assay of percutaneous absorption is using diffusion chamber made from glass and filled with animal/human living or dying skin(11). The method using diffusion chamber is simple, rapid and accurate, but it has a trouble in handling the radiolabelled sample and a difficulty in measuring correctly the amount of radiolabelled compounds on assay of percutaneous absorption. By contrast, it is known that confocal laser scanning microscopy(CLSM) can provide more significant improvement of resolution in measuring a precise quantitative percutaneous absorption of samples(12).

II. Materials & Methods

1. Material

MLV liposome were prepared by the following method ; After mixing 5.0ml glycerin, 9.0ml amphiphilic lipid and 58.7ml distilled water with a mixer during heating at 95-100℃, and added the mixture of 2.0ml propylene glycol, 0.2ml methyl paraben, 0.1ml chlorophenesin and 5.0ml sodium lactate methyl silanol, stirred at 70℃. then this emulsion was passed through a microfluidizier and after cooling until 40℃, 20ml HEE was added. And cooled at 30℃.

2. Preparation of rat skin and application of MLV

For animal test of percutaneous absorption 11-week-old, hairless rat(250-300g) was used, purchased at 4-weeks old and reared at an animal breeding ground for 7 weeks. Rat was reared at 23± 1℃, and a humidity of 60± 5%. After rat was put under anesthesia using injection of 0.5ml rompum and keramin diluted with 0.85% sodium chloride under the abdominal cavity, 0.14g cream-type MLV was applied in two circles of 3cm diameter(28㎠) on the back skin, with variable concentration(5%,10%, 20% and 90%),

and the site of application was covered with Tegaderm for 24hr. Also skin samples were collected at 3hr, 6hr, 12hr, 24hr at which time background level of percutaneous absorption was approached. After rats were sacrificed at this time, 1㎠ split-section was placed in OCT(Ornithine Carbamyl Transferase) compound (Tissue-Tek) and frozen at -20℃. Frozen tissue(30μm thickness) was cut with a cryostat and at once examined under the confocal laser scanning microscope(CLSM510, Carl Zeiss).

3. Analysis of MLV structure

In order to measure the MLV's size and shape, MLV's membrane was labelled by NBD C_{12}-HPC(7-Nitrobenz-2-oxa-1,3-diazol-4-yl(NBD)-conjugated phosphatidylcholine), final concentration of 20μM, was analyzed by CLSM using at X1,000 of lens and X3 zooming(final magnification of X3,000) and also, we observed MLV using TEM (Transmission electron microscope) at X20,000.

4. Analysis of MLV stability

To evaluate of stability of cream-type MLV, we observed image of MLV on slide glass using CLSM. The fluorescence of Hibiscus Esculentus Ext.(HEE) was determined at excitation 396nm and emission 466 nm.

5. Analysis of MLV percutaneous absorption

In experiment in which MLV containing HEE on permeation was measured, 90% MLV in cream was applied to the surface of rat skin and the fluorescent light of HEE in MLV was determined by CLSM. Also skin samples were collected at 5%, 10%, 20%, 90% at which concentration background level of percutaneous absorption of each tissue layer, such as stratum corneum, epidermis and dermis, was approached. In dermis, percutaneous absorption was measured only until 500μm from the outside, using HEE in MLV as a marker. Also skin samples treated with 90% cream-type MLV was collected at 3hr, 6hr, 12hr, 24hr at which time background level of percutaneous absorption were approached and were analyzed by CLSM.

6. Comparison with the percutaneous absorption of three cream-type containing HEE

We had prepared three cream-type containing HEE; 85% HEE(in cream), 20%

liposome-type HEE(final conc. 8%) and 20% MLV-type HEE(final conc. 8%). These were measured comparatively in 11-week-old hairless rat. Skin samples were collected at 12hr and were viewed by CLSM.

7. The quantitative analysis and observation using CLSM(Confocal laser scanning microscope)

Split-section of rat skin was measured by confocal microscope with X63 water-immersion objective lens, 488nm Ar laser and 543 HeNe laser and the scanned image was defined by LP560 and BP505-530 emission filter. An image memory was used to determine the fluorescence analysis by image processing program. The fluorescence of HEE in MLV's water phase was analyzed by HeNe laser providing the light of 543nm. The spectrum of HEE excitation and emission were shown in Fig 1, identified by spectrofluorometer(DMX-1000, SLM AMINCO). In HEE, the fluorescence has a maximum excitation at 396nm and the emission is centered on 396nm. But emission spectrum at 396nm, UV region, is unsuitable to analysis using confocal microscope, so emission spectrum at 488nm and 543nm of visible region was investigated. In HEE, much higher value of emission was observed at 543nm than at 488nm, showing Fig. 2. Therefore image of HEE using CLSM(LSM510, Carl Zeiss) was analyzed by 543nm HeNe laser using TRITC.

III. Results & Discussions

1. Analysis of structure of MLV

1) Analysis by confocal laser scanning microscopy

As shown in Fig. 3, the image of suspension-type MLV observed by CLSM was small particle and the particle size was 0.3-0.5μm mostly. Because of very small particle size and difference of fluorescence intensity, CLSM was not suitable for identifying of MLV shape precisely.

2) Analysis by TEM

Most MLV observed by TEM was globular shape(Fig. 4). Being similar above result, the particle size of MLV was 0.3-0.4 μm, TEM was more suitable rather than CLSM in order to observe small size MLV.

3) Stability of cream-type MLV

The result of stability of cream-type MLV observed by CLSM was shown in Fig.5. 2 weeks later, fluorescence intensity of cream-type MLV was slightly weaker than the initiative state. From this result, we found that cream-type MLV was degraded slowly.

2. Penetration of MLV

1) Observation of penetration degree of MLV

As shown in Fig. 6, the most external layer of skin that of treated MLV has been much stronger fluorescence intensity than non-treated MLV. Also, we could observe fluorescent light in the most external layer of non-treated skin, but it means there are auto-fluorescence materials in the external layer of non-treated skin.

Continuously, we investigated from external layer to muscle layer in tissue, MLV treated skin had stronger fluorescence intensity than MLV non-treated skin. This means that HEE within MLV penetrated into muscle layer of skin. But we have questions about how it penetrates into skin by HEE within MLV. We made proposal of two possibilities about mechanism of penetration to skin, one is that active ingredient contained MLV is directly penetrate into tissue through MLV incorporate with external cells in tissue, and the other is that MLV is directly penetrated into skin.

To prove these mechanisms, we need to understand about characteristics of active ingredients within MLV and UV technique more detail.

2) Effect of MLV concentration on penetration

As shown in Table 1, it shows similar result in penetration degree between 5% MLV and control. When treated 10% MLV to skin, both external and internal skin were increased over two-fold than control. But in case of over 20% MLV, it makes little difference with treatment of 10% MLV. It means that skin penetration of MLV has optimal concentration on penetration. We concluded that 10% MLV was most optimal concentration when considering skin penetration.

3) Analysis of incubation time of cream-type MLV on the skin penetration

As shown Table 2, when rat skin was treated with 90% MLV for 3 hr, the fluorescence intensity of dermis and epidermis was slightly increased than control. Actual penetration was observed after 6hr and the degree of penetration in skin was similar during 6hr-12hr. But penetration in epidermis increased twice after 6hr and 3.4 times after 12hr, and the degree of penetration in dermis was similar with 6hr and 12hr. Therefore, the optimum penetration time was 6hr -12hr.

4) Comparison with penetration ability of 3 cream-type containing HEE

The result of penetration ability of 3 cream-type containing HEE on the skin penetration was shown in Table 3. Strong auto-fluorescence intensity was observed on the stratum corneum of control. But weak auto-fluorescence intensity was observed on epidermis and

dermis of control. We detected stronger fluorescence intensity on the HEE-cream than that of control in epidermis. But there is no difference in dermis. A similar effect on penetration of epidermis of liposome-type HEE and HEE-cream was observed. We found stronger penetration of MLV-type HEE than that of HEE-cream in epidermis and dermis but the penetration of HEE-cream was similar in some tissues.

Generally, we observed stronger effective on penetration of MLV-type HEE than that of liposome-type and HEE-cream in epidermis and dermis of hairless rat skin

IV. Conclusion

1. MLV was globular shape and the range of particle size was 0.3-0.5μm mostly.

2. Cream-type MLV had high stability comparatively.

3. When treated with MLV to rat skin, skin penetration was enhanced, especially, the optimum concentration of MLV on penetration to rat skin was 10%.

4. The optimum penetration time on MLV to hairless rat skin was 6hr-12hr.

5. MLV-type HEE was more effective on percutaneous absorption than HEE-cream or liposome-type HEE.

V. References

1. Elias, P.M. and Feingold, K.R., Lipids and the epidermal water barrier : Metabolism, regulation, and Physiology, Seminar in Dermatology, 1992; 11: 176-182.

2. Hueber, F., Schaefer, H. and Wepierre, J., Importance of transdermal and transfollicular routes in percutaneous absorption in man : Demonstration with steroids, IFSCC Congress Yokohama(Poster), 1992.

3. Hans, S. and Thomas, E.R., Skin barrier, Karger, 1996.

4. Mezei, M. and Gulasekharam, V., Liposome a selective drug delivery system for the topical route of administration : Gel dosage form, J. Pharm. Pharmacol., 1982; 34: 473-474.

5. H. Kikuchi, and K. Inoue, Liposomes I, (No. 9), 1983.

6. Reifenrath, W. G., Chellquist, E.M. Shipwash, E.A. Jederberg W.W. and Krueger G.G., Percutaneous penetration in the hairless dog, weanling pig, and grafted athymic nude mouse : Evaluation of models for predicting skin penetration in man, Br. J. Dermatol., 1984; 111(Suppl. 27):123-135.

7. Wester R.C. and Maibach, H.I., In vivo animal models for percutaneous absorption, Marcel Dekker, 1989.

8. Rougier, A., Lotte, C. and Maibach H.I. The hairless rat : A relevant in vivo : Comparison in rat, rabbit, pig, and man, J. Invest. Dermatol. 1987; 88 : 557-581.

9. Bronaugh, R.L. and Stewart, R.T., Methods for in vitro percutaneous absorption studies. V. Permeation through damaged skin, J. Pharm. Sci., 1985; 74: 1062-1066.

10. Hsieh, D.S., Drug preparation enhancement, Marcel Dekker, 1994.

11. Gummer, C.L., Hinz, R.S. and Maibach, H.L., The skin penetration cell : A design update, Int. J. Pharmaceutics, 1987; 40: 101-104.

12. Shotton, D. M. Confocal scanning optical microscopy and its applications for biological specimens, J. Cell Sci., 1989; 94: 175-206.

Table 1. Effects of cream-type MLV concentration on rat skin penetration.

MLV concentration	Stratum Corneum	Epidermis	Dermis distance from epidermis(μm)		
			0-100	100-310	310-520
0%	237	26(1.0)	35(1.0)	27(1.0)	19(1.0)
5%	240	48(1.8)	43(1.2)	28(1.0)	33(1.7)
10%	203	62(2.4)	74(2.1)	68(2.5)	55(2.9)
20%	228	69(2.7)	57(1.6)	43(1.6)	34(1.8)
90%	260	80(3.1)	54(1.5)	55(2.0)	51(2.7)

Table 2. Effects of incubation time of cream-type MLV on skin penetration.

MLV Incubation time	Epidermis	Dermis distance from epidermis(μm)		
		0-100	100-310	310-520
0 hr	26(1.0)	35(1.0)	27(1.0)	19(1.0)
3 hr	32(1.2)	37(1.1)	33(1.2)	28(1.5)
6 hr	52(2.0)	51(1.5)	48(1.8)	38(2.0)
12 hr	88(3.4)	55(1.6)	40(1.5)	42(2.2)
24 hr	80(3.1)	54(1.5)	55(2.0)	51(2.7)

Table 3. Comparison of the penetration ability of 3 cream-type containing HEE

Distance From Surface	Control	Only HEE	Liposome Type-HEE	MLV-type HEE
Stratum corneum(15μm)	220(1.0)	253(1.2)	210(1.0)	249(1.1)
Epidermis (15-50 μm)	83(1.0)	133(1.6)	118(1.4)	232(2.8)
Dermis (50-130 μm)	51(1.0)	47(0.9)	49(1.0)	79(1.5)

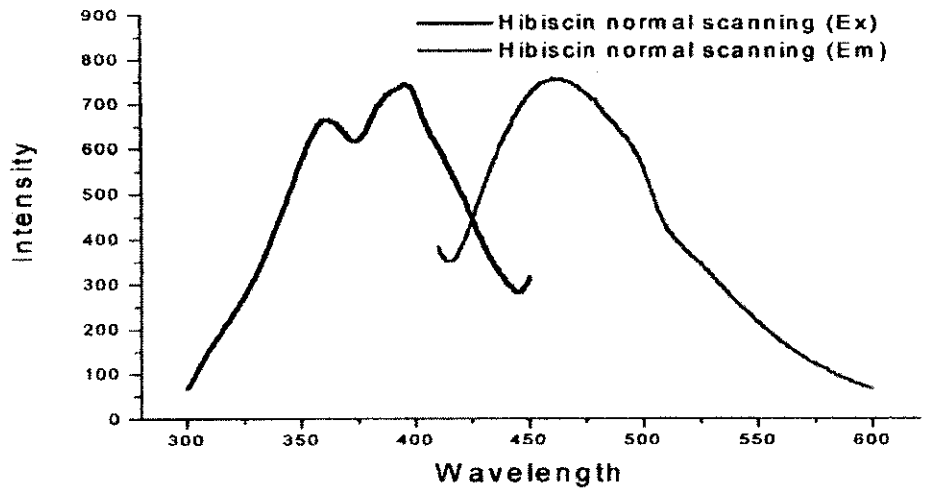

Fig. 1. Excitation(Ex) and emission(Em) spectrum of HEE analyzed by spectrofluorometer.

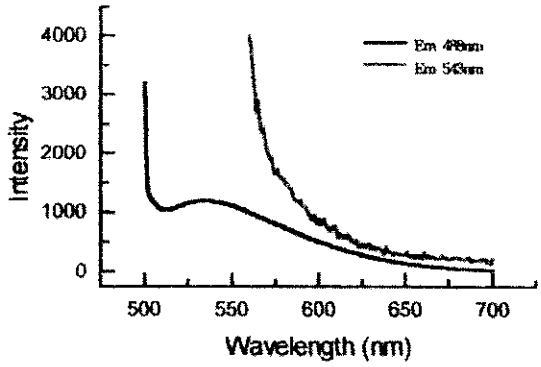

Fig. 2. Emission (Em) spectrum of HEE at 488 and 543 nm.

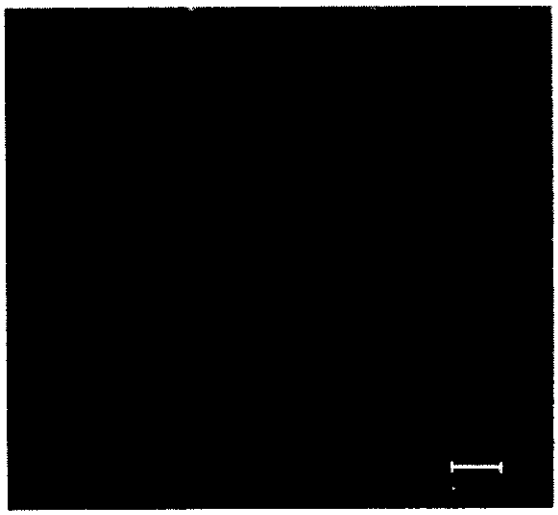

Fig. 3. Suspension-type MLV observed by CLSM (X3,000)

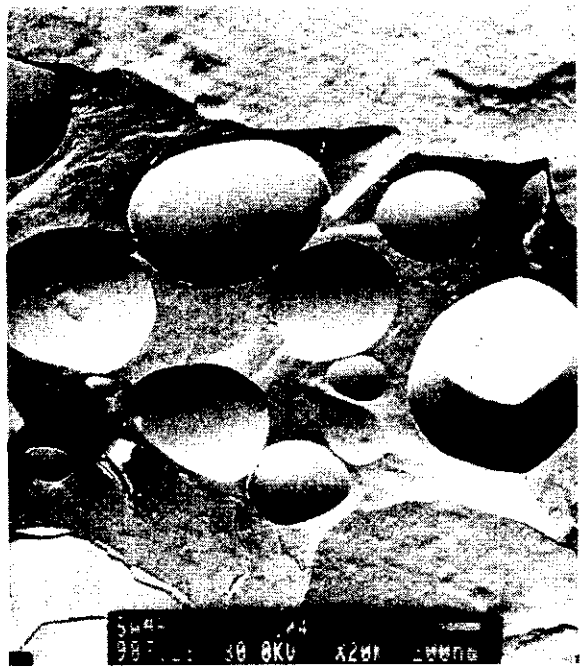

Fig. 4. Cream-type MLV observed by TEM (X 20,000)

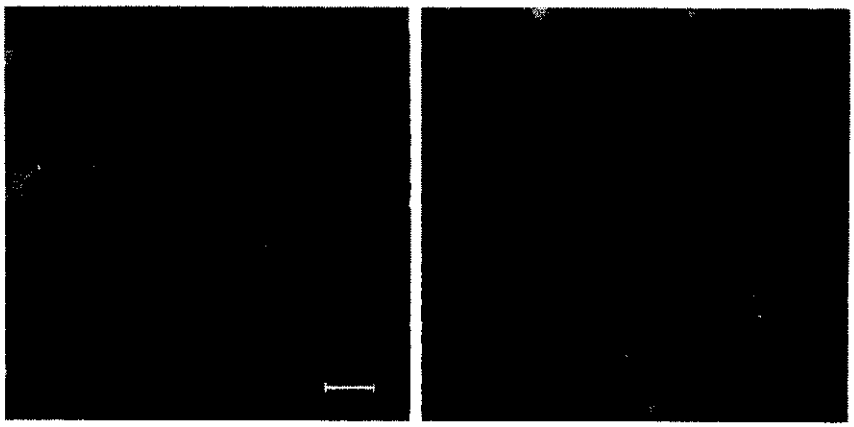

Fig. 5. Stability test of Cream-type MLV. Cream-type MLV observed at 0(left) and 14(right) days-using CLSM.

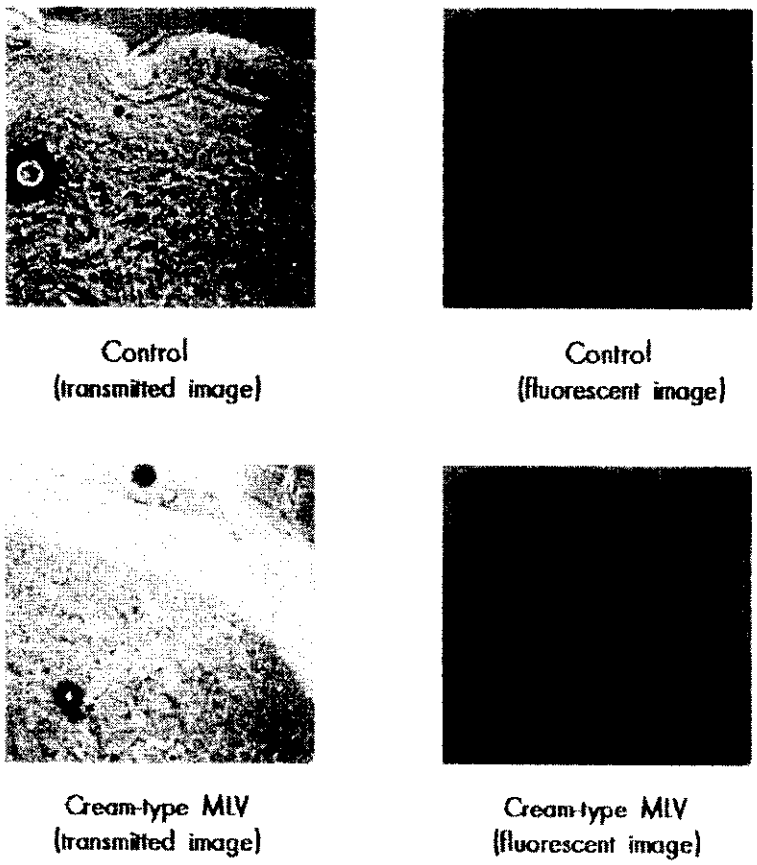

Fig. 6. The penetration of Cream-type MLV into skin of hairless rat observed by CLSM.

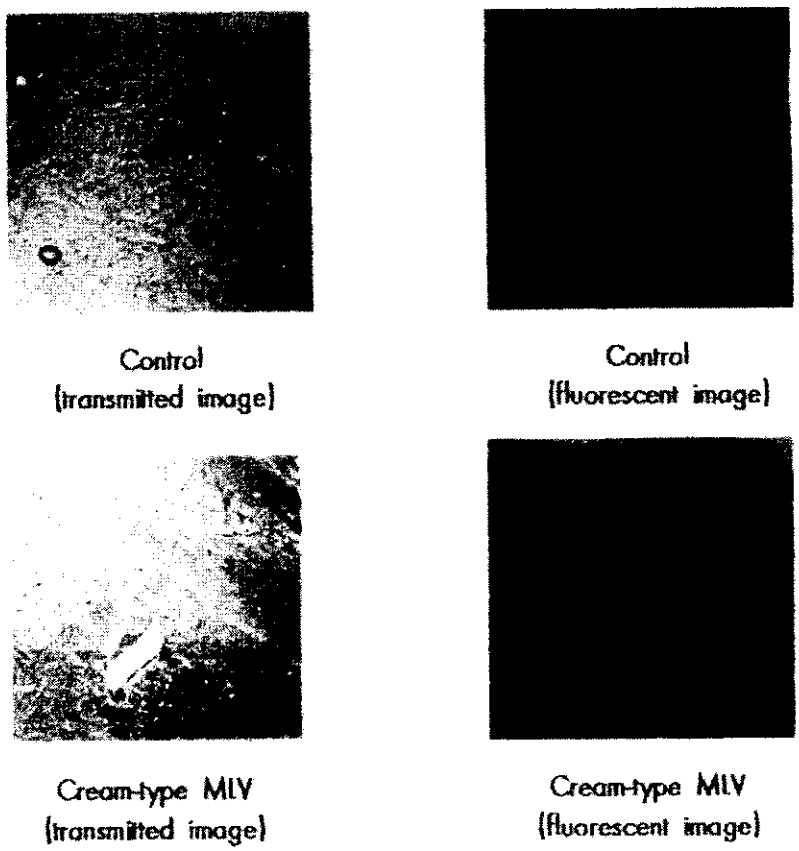

Fig. 7. The penetration of Control and 10% MLV into stratum corneum (SC) of hairless rat skin.

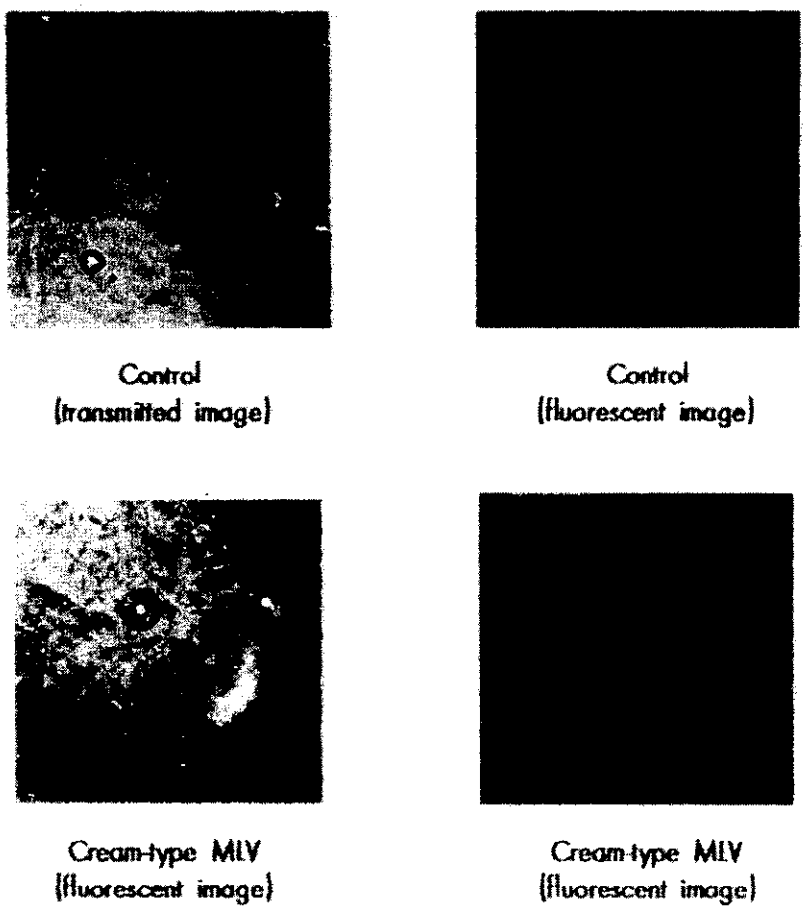

Fig. 8. The penetration of Control and 10% MLV into epidermis of hairless rat skin

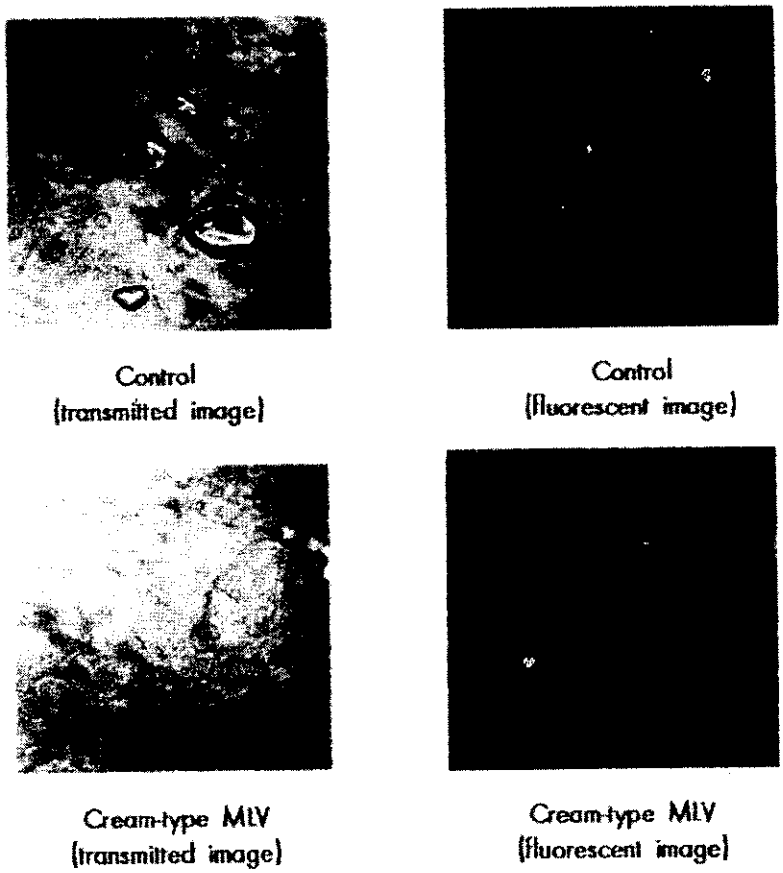

Fig. 9. The penetration of Control and 10% MLV into dermis of hairless rat skin.