Improvement of Epidermal Barrier Properties in Cultured Skin Substitutes after Grafting on Athymic Mice

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Running head: Barrier properties of skin substitutes.

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Abstract

Barrier function in cultured skin substitutes prepared from human cell sources was measured by non-invasive (surface hydration, transepidermal water loss) and invasive methods (water penetration, niacinamide flux) pre and post grafting onto athymic mice. In vitro measurements were made on day 7 and day 14. Although barrier function dropped tremendously from day 7 to 14, the values obtained were still far from those obtained with native human skin controls. Additional cultured skin substitutes were grafted on athymic mice on day 14, and skin was harvested 2 weeks and 6 weeks post grafting. Grafting brought about a substantial decrease in all measurements by 2 weeks and almost completes normalization of barrier function after 6 weeks. The most sensitive measure of this recovery was niacinamide permeability, which decreased from $280 \pm 40\times10^{-4}$ cm/h in vitro to $17 \pm 30 \times10^{-4}$ two weeks post grafting and $5 \pm 2\times10^{-4}$ cm/h six weeks post-grafting, versus control values of $2 \pm 1\times10^{-4}$ cm/h in human cadaver skin and $0.6 \pm 0.4\times10^{-4}$ cm/h in human epidermal membrane prepared from freshly excised breast skin.

Abbreviations: CSS – cultured skin substitutes; NHS – native human skin; NMS – native mouse skin; SC- stratum corneum; TEWL – transepidermal water loss; HCS- human cadaver skin; HEM – human epidermal membrane

Introduction

Stratum corneum (SC), the outermost layer of human skin, is a highly effective barrier membrane protecting the body from entrance of infectious substances and loss of water and necessary nutrients. Restoration of this barrier following a wound or skin damage is of great significance to the general well being of the body. In recent years, skin substitute models consisting of epidermal and dermal layers of skin have been developed as alternative treatment modalities for large skin wounds due to their ability to restore epidermal barrier. These models have been developed by a variety of techniques including growing keratinocytes on acellular or de-epidermized dermis, [1, 2] collagen matrices, [3] and inert filters [4]. They exhibit morphological and biochemical features very close to those observed in native tissue. This has led to their increased use as adjunct therapies for treatment and healing of various full thickness skin loss conditions, including burns, congenital giant nevus, plastic/cosmetic surgery and chronic wounds [5, 6]. The success in the clinic has led scientists to believe that these models could be considered as a feasible alternative to replace the traditional skin models in toxicological and pharmacological studies in vitro. Recently these models have been used for short term cytotoxicity assays [7] and skin metabolism studies [8]. However their use in vitro is still limited due to deficient barrier function. Skin penetration measurements have shown a vast difference in permeability between native human skin (NHS) models and cultured skin models in vitro, [1, 9-13] including cultured skin substitutes (CSS) from our laboratories [14]. CSS consists of cultured human epidermal keratinocytes attached to an implantable collagen-glycosaminoglycan substrate populated with dermal fibroblasts [15]. Modifications to the culture media [16-19] and growth conditions [20] have restored much, but not all, of the anatomy and physiology of NHS. These are manifested in improved SC development and barrier

function. However certain deficiencies are still seen in all skin substitutes, in terms of epidermal differentiation, lipid content, and absence of desquamation [21].

CSS grafted onto athymic mice have shown the ability to generate a well differentiated epidermis [16]. The abnormalities seen in the lipid profile are also restored, leading to a more competent SC barrier [22]. Barrier function has been assessed using non-invasive biophysical measurement of surface hydration in vitro and post grafting [23]. Post grafting capacitance values obtained were comparable to NHS. Higounenc et al. [24] saw similar improvements in barrier properties after grafting reconstructed human epidermis onto nude athymic mice. They performed non-invasive transepidermal water loss (TEWL) measurements along with $^{3}H_2O$ penetration measurements, and established that the SC of their reconstructed epidermis forms an impermeable protective barrier in vivo. Thus, transplantation of CSS onto athymic mice would seem to provide a valuable model for identification of factors that contribute to normalization of skin functions after grafting. This approach also enables us to study the development of fully functional epidermis under conditions in which the essential nutrients are provided by the host. This may provide valuable insights on modifications to be made in nutrients and other culture conditions in vitro.

Although surface hydration and TEWL are standard biophysical measurements used to assess barrier function in vivo, their validity in vitro is controversial [25]. Measurement of tritiated water flux has been considered a standard procedure to measure the barrier integrity and is widely used on other in vitro skin models [26-28]. The objective of this study was to determine the effect of grafting on development of the SC barrier in CSS. This was accomplished via measurement of the penetration of standard compounds along with the non-invasive determination of barrier function.

Preliminary studies of CSS permeability showed a decrease in permeability post grafting vs. in vitro [29]. However due to limited sample size, a time course measurement was not possible. This study establishes the time course of barrier development. In addition to water flux, niacinamide penetration was also studied. Surface hydration and TEWL measurements were also conducted. A method to measure TEWL in vitro was developed which took into account controlled atmospheric conditions.

Materials and Methods

Preparation of CSS: This process was a three step procedure described extensively in the literature [15, 30]. A collagen-glycosaminoglycan substrate was prepared and seeded on subsequent days with human fibroblasts and keratinocytes (culture day 0). The human skin cells were obtained from enzymatic digestion of adult surgical discard tissue. CSS were cultured at the air-liquid interface (5% CO_2, 37°C) to allow for differentiation and formation of the SC, and were maintained in these conditions throughout the incubration period. Four large CSS (~ 300 cm^2) were prepared, and on culture day 7 CSS were cut into quarters (~ 50 cm^2) to facilitate in vitro data collection and grafting on mice.

Human epidermal membrane (HEM): Fresh, full thickness skin was obtained from bilateral reduction mammoplasty (Department of Plastic Surgery, College of Medicine, University of Cincinnati). The underlying fat was trimmed off. The dermis was separated from the epidermis by suspension of cut pieces in distilled water for 2 min at 60°C [31].

Human Cadaver Skin (HCS): Cryoprotected, cadaveric, split-thickness human skin specimens (stored in 10% glycerol) were obtained from Ohio Valley Tissue and Skin Center (Cincinnati, OH) and stored at -80°C until use. These samples were obtained with a dermatome and had a nominal thickness of 300 μm.

Murine Skin: Native murine skin (NMS) was used as a control. Skin was harvested from the torsos of athymic mice after they were euthanized. These studies and the grafting studies

described below were conducted with the approval of the University of Cincinnati Institutional Animal Care and Use Committee and according to principles of laboratory animal care established by the NIH.

Experimental time line: Surface hydration, TEWL, water penetration and niacinamide flux were measured on days 7 and 14 in vitro. On culture day 14 CSS were grafted onto athymic mice and biopsies were collected for light microscopy. Two and six wks post grafting the mice were euthanized, the grafts harvested samples collected (n = 1/time point) for histological evaluation and all barrier function measurements except surface hydration were made ex vivo. Surface hydration was measured more frequently both in vitro and in vivo. HEM, HCS and NMS were used as controls. Surface hydration and TEWL values of NHS from the literature were used as the in vivo control.

Grafting of CSS onto athymic mice: On culture day 14, CSS (n = 9) were grafted orthotopically to 2×2 cm full thickness skin wounds prepared surgically in athymic mice (nu/nu; Harlan, Indianapolis, IN). Wounds were prepared leaving the panniculus carnosus intact and the CSS (~4 cm^2) were sutured in place with an overlay of nonadherent dressing (N-Terface; Winfield Laboratories, Richardson, TX). The wounds were dressed as previously described [32]. The mice were euthanized and the grafts harvested, 2 wk and 6 wk post grafting.

Histological evaluation: CSS biopsies collected for light microscopy were fixed with 2% glutaraldehyde/2% paraformaldehyde for a minimum of 1 h. Samples were then processed, embedded in glycol-methacrylate resin, sectioned and stained with toluidine blue using standard

techniques. Sections were examined using a Nikon Microphot-FXA microscope (Nikon, Inc., Instrument Group. Melville, NY) and photographed using a Spot-Jr. Digital Camera (Diagnostic Instruments, Sterling Heights, MI).

Surface hydration measurements: Measurements of surface electrical capacitance were made with an impedence-based NOVA^{TM} Dermal Phase Meter (DPM 9003; NOVA Technology Corp., Gloucester, MA) connected to a portable computer that recorded 10 serial readings at 1-second intervals [23]. The final value collected after 10 seconds of sampling was used to determine surface hydration levels.

Transepidermal water loss measurements: A DERMALAB® evaporimeter (Dermalab Inc., Philadelphia, PA) was used to measure water loss in vitro. On the days of barrier measurements, the CSS were mounted on modified Franz cells (0.79 cm^2) which were magnetically stirred and maintained at 37°C in a thermostatted block [33]. The receptor solution was Dulbecco's phosphate buffer saline (PBS), pH 7.4, containing 0.02% sodium azide. After a 1 h equilibration, the DERMALAB probe was placed on the short (0.5 cm) donor chamber opening. A continuous measurement was taken for 1 min, and the 20 s mean was reported. Although this method does not permit direct contact with the skin, a proper seal was obtained between the Franz cell and the probe.

Water permeation: The method has been described previously [26, 28]. The protocol provides a permeability screen without exposing the tissue to prolonged hydration. Following the TEWL measurements, 150 μL of ^3H_2O, specific activity 0.4 μCi/mL, was applied to the epidermal

surface. After 5 min the tissue was blotted dry with a cotton swab. At 60 min post-dose the receptor chamber contents were removed for scintillation counting and replaced with fresh buffer. Residual $^{3}H_2O$ was thoroughly rinsed from the tissue by several receptor fluid exchanges and an overnight equilibration.

Niacinamide permeation: The low oil solubility and high water solubility of niacinamide (Figure 1) make it an extremely sensitive probe of stratum corneum barrier function. The donor chamber was filled with 0.5 mL of buffer containing ^{14}C-niacinamde (1 μCi/mL) and sufficient unlabelled niacinamide to achieve chemical concentration of 10 μg/mL and a dose of 6.3 μg/cm^2. Both radioactive and unlabelled niacinamide were obtained from Sigma Chemicals (St Louis, MO). Radiochemical purity of the ^{14}C-niacinamide was stated by the manufacturer to be ≥95 %. The receptor solutions were exchanged periodically with fresh buffer and analyzed for radioactivity by liquid scintillation counting. Ultima Gold™ liquid scintillation fluid was purchased from Packard Instrument Company (Downers Grove, IL). Mean penetration and its standard deviation were determined for each time point following a near logarithmic transformation to remove skewness [28].

Results

Histological Evaluation: Morphology of CSS extracted from athymic mice 6 wks post grafting were compared to the CSS appearance at the time of grafting (day 14 in vitro). By day 14 the CSS showed a presence of a fully differentiating epidermis including a well developed SC analog (Figure 2a). At 6 wk post grafting the CSS structure and organization was very similar to NHS and more improved than that seen at day 14 in vitro. All epidermal strata were well defined. The basal layers were well formed and showed the presence of viable and replicating keratinocytes. These layers were also well attached to the dermal layer. The SC was not as thick as at day 14, indicating normalization of the desquamation process post grafting (Figure 2b).

Barrier measurements on control tissues: HEM, HCS and NMS were used as reference controls. The barrier measurements made on these tissues are tabulated in Table 1.

Surface hydration: In vitro formation of epidermal barrier in CSS was seen by the continuous drop in capacitance values from day 5 to day 14 (Figure 3). The capacitance values at day 14 were two- fold higher than those for NHS in vivo (Figure 3). By 2 wks after grafting, the SEC values were lower than those seen in vitro and were the same order of magnitude as NMS. By 6 wks the capacitance fell further (127 ± 31) DPM units and became comparable to NHS in vivo.

Transepidermal water loss: TEWL values were high and fairly constant during the in vitro formation of epidermal barrier (Figure 4). By day 14 in vitro the values for TEWL were still 3- to 4-fold greater than values of NHS in vivo and HCS in vitro. However there was a rapid

restoration of epidermal barrier following grafting as demonstrated by the considerable decrease in TEWL 2 wks after surgery. These values were very similar to both NHS and HCS ex vivo, and at 6 wks they were similar to values of NMS ex vivo. These results indicate that CSS on grafting forms a barrier to transepidermal water loss similar to that seen in NHS in vivo, 5-10 $gm^{-2}h^{-1}$ [34, 35].

$^{3}H_2O$ penetration: Figure 5 shows the results of $^{3}H_2O$ penetration studies on CSS in vitro and ex vivo. As was seen in surface hydration measurements, there was a considerable drop in water permeability from day 7 to 14. At day 14 the barrier was still highly permeable - almost 18-fold higher than HCS. This barrier was restored on grafting. By wk 2 ex vivo, the grafts were approximately 3 fold more permeable than HCS. Further improvement was seen by wk 6 with $^{3}H_2O$ penetration values (0.3 ± 0.1) lower than those obtained with freshly prepared HEM. By this measure the CSS developed a competent SC permeability barrier on grafting.

^{14}C-Niacinamide penetration: Figure 6 shows representative plots of ^{14}C-niacinamide penetration in vitro and ex vivo. For the in vitro samples steady state flux was attained within minutes of application of the drug solution. A decline in flux was observed after 4-8 h due to the significant decrease in drug concentration in the donor chamber. In contrast no significant drug depletion from the donor chamber was observed in the ex vivo samples and a reasonable steady state flux was maintained for 48 h.

Steady state flux is determined from the linear regions of the plots in Figure 6. This flux was converted into permeability coefficient k_p by the relation

$J_{ss} = k_p/C_v$

Permeability coefficient is plotted as a function of time in Figure 7. From day 7 to day 14 the permeability dropped approximately 6-fold. At 2 wk ex vivo, the permeability was 10-fold lower than day 14 in vitro and at 6 wks ex vivo permeability was 40-fold lower.

Discussion

An intact and fully functional skin barrier is of great significance to human health and would be valuable to most in vitro cutaneous studies. Despite similarity in morphology and composition, CSS in vitro exhibit a significantly impaired barrier function with respect to NHS. In this study, four methods of barrier assessment were conducted – surface hydration and TEWL (non invasive) and $^{3}H_2O$ and niacinamide penetration (invasive).

All measurements confirmed that the CSS forms a transient barrier in vitro, which is at its optimum function at day 14. This is consistent with previous studies from our laboratories and corresponds to clinical application of CSS within 2 wks of keratinocyte inoculation [23]. Therefore day 14 in vitro was chosen as the appropriate time for grafting the CSS onto mice. Earlier work has shown that formation of a functional barrier in vitro leads to faster wound closure and vascularization post grafting [16]. Grafting of CSS onto athymic mice resulted in rapid and substantial improvement in barrier function by all measurements.

Human skin grafted onto athymic mice has long been considered to be a useful model for understanding processes related to epidermal proliferation and differentiation [36-38]. More recently skin substitutes grafted onto athymic mice have led to further development of this in vivo approach due to the improved differentiation, epidermal lipid structure and SC barrier exhibited post grafting [23, 24, 39]. Pouliot and coworkers observed histological similarities between reconstructed human skin grafted onto athymic mice for 21 days and NHS [39]. The reconstructed human skin showed 10-15 cell layers of a well differentiated epidermis. Higounenc et al. also observed an improvement in epidermal differentiation and barrier function in reconstructed human skin after grafting [24]. Barrier function improvement in vivo was

reported following normalization of lipid composition and structure after 1 to 4 months of transplantation. Previous work in our laboratories has shown a normalization of lipid composition and structure in the CSS over a prolonged time frame (6-24 months) post grafting [22].

SC lipids constitute the main permeability barrier in the skin. Hence normalization of lipid structure and composition in vivo suggest a corresponding improvement in permeability barrier. Higounenc et al. observed a 3-fold drop in flux through the reconstructed epidermis from day 21 in vitro to week 6 ex vivo. At 6 wks post grafting the flux values were similar to those seen in NHS. Our method to measure ^3H_2O penetration was different and has been used previously to study skin integrity of cadaver or freshly excised human epidermal membrane (HEM) [26, 28]. ^3H_2O penetration dropped rapidly after grafting, and at 6 wks post grafting was in the same range as HEM (0.57 ± 0.01) and lower than HCS. HEM is a more stringent standard for skin barrier measurement in comparison to HCS. Hence the CSS 6 wk post grafting met high standards of skin integrity.

In addition to water penetration we studied the penetration of niacinamide. The flux range reported in our laboratories was 7 - 20×10^{-4} $\mu g/cm^2/h$ for HEM and HCS (Table 1). The low permeability is associated with low lipid/water partition coefficient, limiting transport through the SC lipids. Thus the extremely high flux of niacinamide seen through CSS in vitro could be due to insufficient levels or lack of these essential lipids. Due to the considerable restoration of lipid structure and composition an increased resistance to niacinamide penetration was observed post grafting.

However, unlike water penetration, niacinamide penetration in CSS 6 wks post grafting was still approximately 3-7 fold higher than the native skin models. This observation corresponds with

the fact that there are still differences in the organization of SC lipids in epidermal grafts as compared to native human skin [21, 22]. Lamellar phase deficiencies have been observed by X-ray diffraction in these epidermal grafts [21]. Niacinamide penetration, which is extremely sensitive to the integrity of the lipid barrier, reflects these differences. Water, on the other hand, is smaller and much more mobile across lipid bilayers; hence $^{3}H_2O$ penetration and TEWL are less sensitive to small defects in the bilayer structure. These properties reached values similar to native skin models by 6 wks post grafting.

Previous studies in our laboratories have shown that the in vivo CSS capacitance measurements reached normal human skin values at 4 weeks post grafting [23]. The present study confirmed these results at 6 weeks post grafting. In this study TEWL measurements were made for the first time in vitro and ex vivo. In vitro the values were very high throughout the culture period. At day 14 in vitro TEWL was 31 ± 11 $g/m^2/h$, which is approximately 3-fold greater than that seen in HCS and NHS. These values are similar to those obtained from superficially damaged skin, or pre term infants at 26-27 wk gestation [40, 41]. Thus CSS may be a useful model to study wounded or premature skin. TEWL values measured ex vivo at 2 wks post grafting (~ 8 $g/m^2/h$) were very similar to NHS in vivo and HCS in vitro. They were about twice those measured by Higounenc et al [24]. Thus the non invasive methods of barrier measurements indicate the formation of a stable barrier in vivo, within a month post grafting.

Thus from the above results we observed that both the invasive and non invasive barrier measurements show tremendous improvement of barrier function post grafting of CSS on athymic mice. HLA-ABC staining of representative healed CSS at 2 and 6 wk after surgery confirmed the engraftment of human keratinocytes. This is consistent with previous studies where direct immunofluorescence staining using a monoclonal antibody against a common

hapten of the HLA-ABC histocompatibility antigen verified that wounds were healed with human keratinocytes [20].

In conclusion CSS are capable of forming a stable and highly resistive barrier in vivo, but the present culture conditions are not yet conducive to support expression of this definitive phenotype in vitro. More modifications to the media formulation and growth conditions are required in order to improve the in vitro barrier properties.

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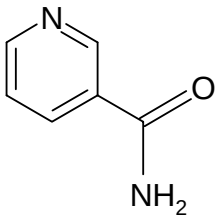
Table 1: Reference values obtained for different control tissue types. (Mean ± SE)

Tissue type	$^{3}H_2O$ Penetration ($\mu l/cm^2$)	TEWL ($g/m^2/h$)	^{14}C-niacinamide permeability × 10^4 (cm/h)
Human Epidermal Membrane	0.57 ± 0.01	n.d.	0.6 ± 0.4
Human Cadaver skin	1.02 ± 0.41	7.7 ± 0.6	2.2 ± 2.2
Native Murine skin	0.60 ± 0.35	0.8 ± 0.6	6.0 ± 1.8

n.d. – not determined



MW 122 Da
mp 130°C
S_w 5×10^2 g/L[a]
Log K_{oct} -0.37[b]

[a]unpublished data from our laboratory
[b]octanol/water partition coefficient from Ref. 38 (low concentration value).

Fig. 1. Structure and physical properties of niacinamide

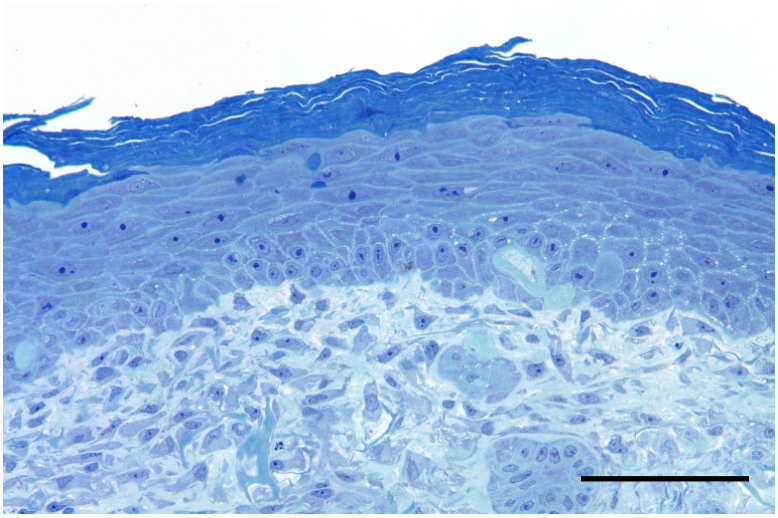


Fig. 2a

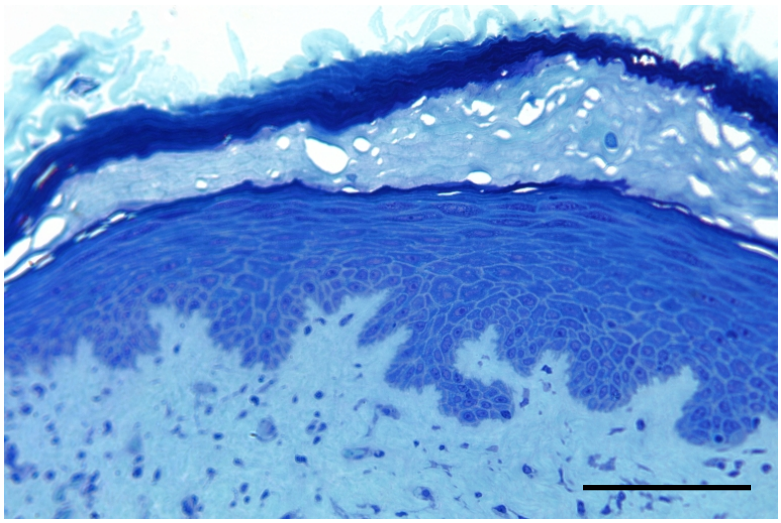


Fig 2b

Fig. 2. Morphology of CSS at the time of grafting (2a) and 6 wks post grafting (2b). Scale =100 μm.

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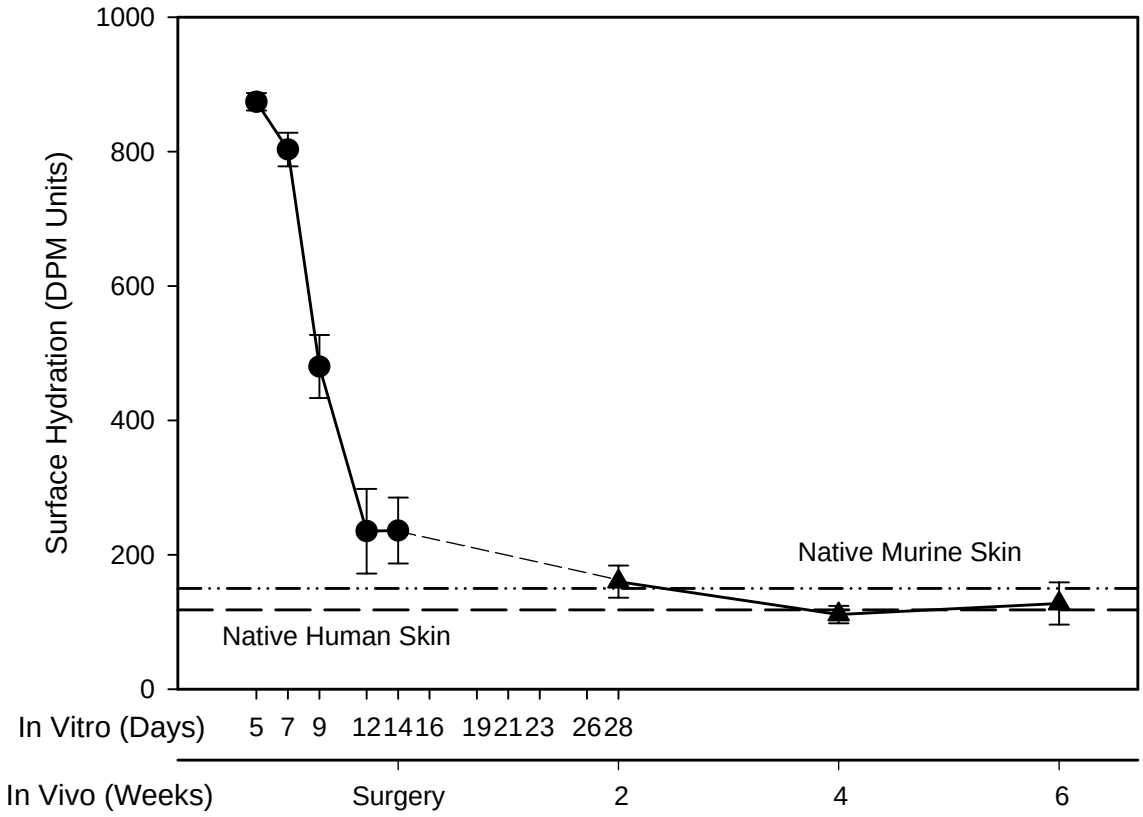

Fig. 3: Surface hydration of CSS in vitro (•) and grafted CSS in vivo (▲) as assessed by Dermal Phase Meter

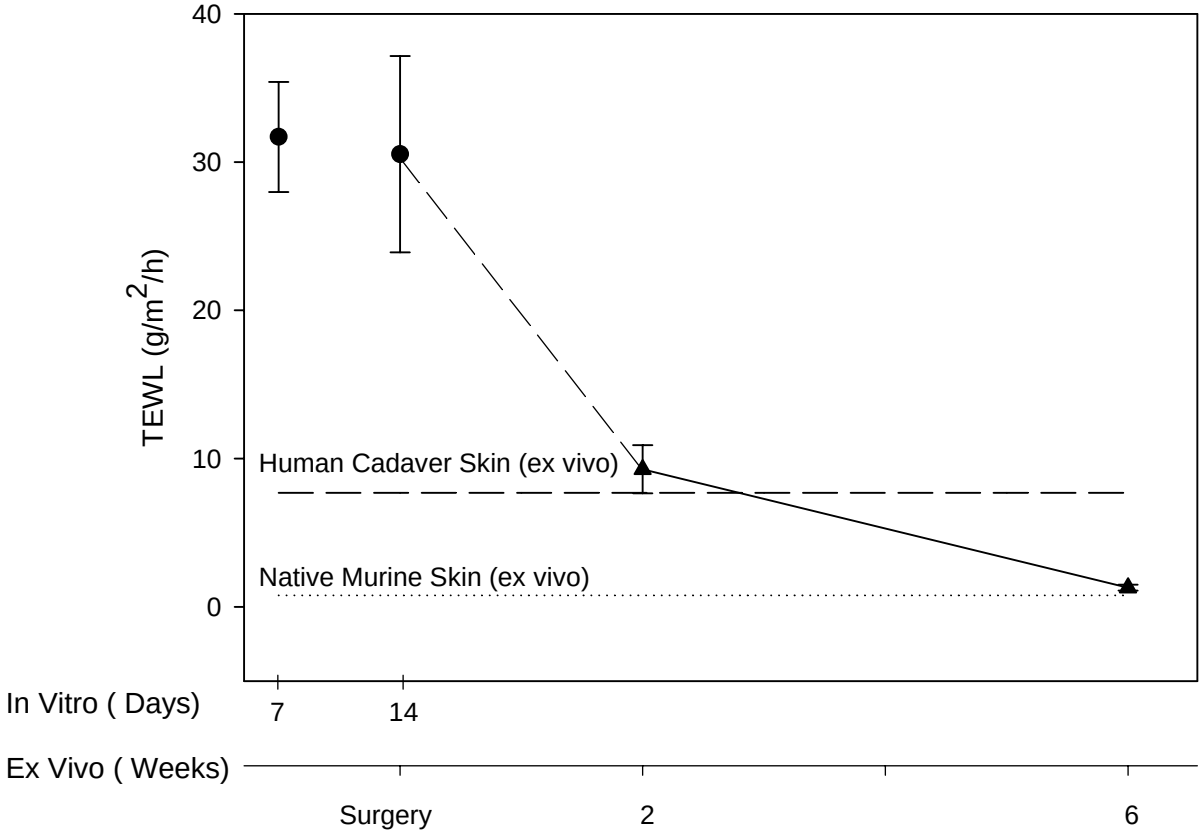

Fig. 4. CSS transepidermal water loss in vitro (•) and ex vivo (▲) (mean ± SE; n = 20-27 in vitro,2-6 ex vivo).The value for human cadaver skin and native murine skin are reference values from Table 1.

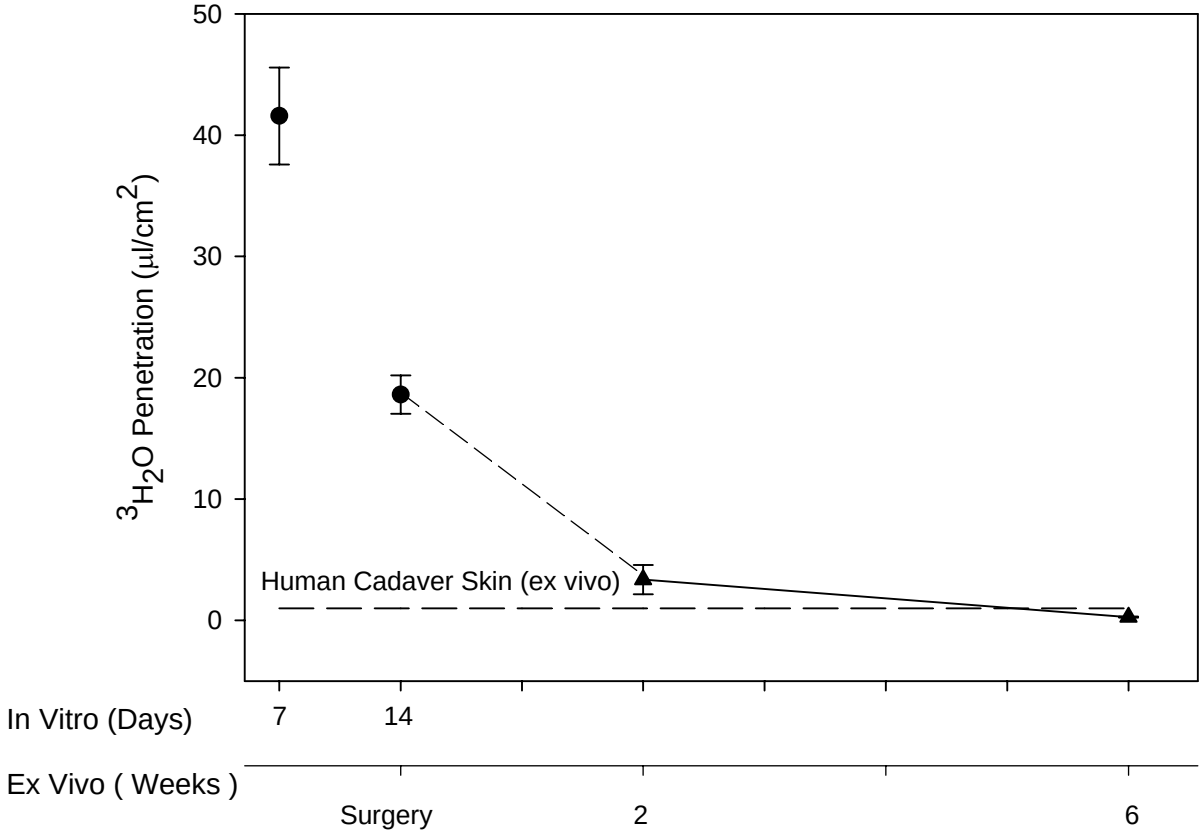

Fig. 5. CSS tritiated water penetration in vitro (•) and ex vivo (▲) (mean ± SE, n = 20-27 in vitro, 2-6 ex vivo). The value for human cadaver skin is the reference value from Table 1.

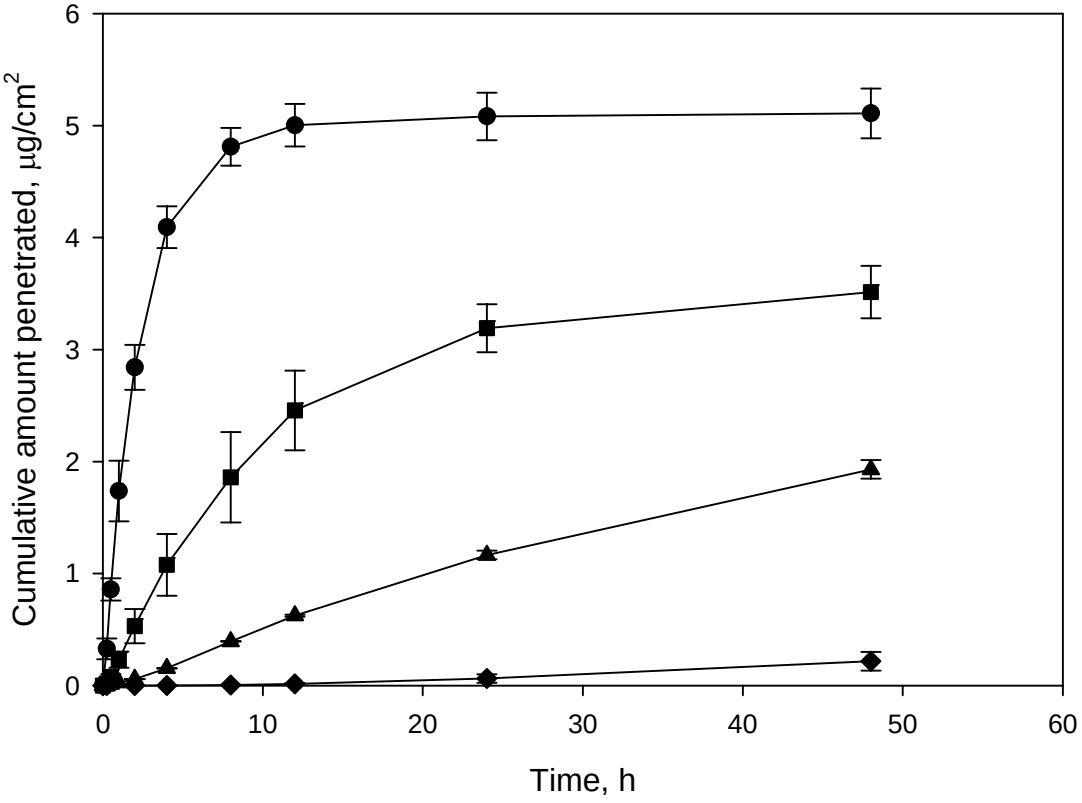

Fig. 6. Representative plots of ^{14}C-niacinamide penetration in vitro and ex vivo (mean ± SE, n = 20-27 in vitro, 2-6 ex vivo). The dose to each cell was 6.3 µg/cm^2 in a volume of 0.5 mL (concentration 10 µg/mL).

The time points of measurements are day 7 in vitro (●), day 14 in vitro (■), week 2 ex vivo (▲) and week 6 ex vivo (♦)

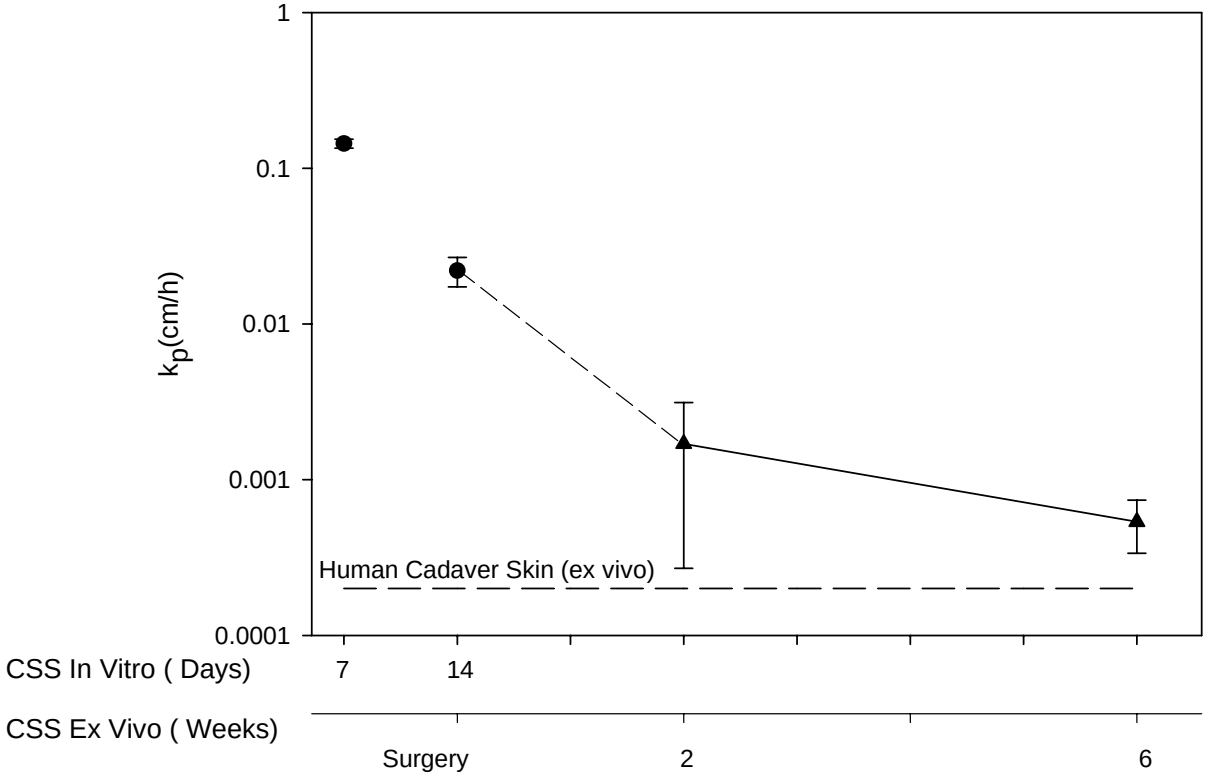

Fig. 7: Permeability of ^{14}C- niacinamide through CSS (mean ± SE) in vitro (•) and ex vivo (▲).

The human cadaver skin value is reference value from Table 1.