Received: 9 November 2021 | Accepted: 3 May 2022

DOI: 10.1111/srt.13165

ORIGINAL ARTICLE

WILEY

Characterization and ex vivo evaluation of excised skin samples as substitutes for human dermal barrier in pharmaceutical and dermatological studies

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Abstract

Background: Excised animal and human skins are frequently used in permeability testing in pharmaceutical research. Several factors exist that may have influence on the results. In the current study some of the skin parameters that may affect drug permeability were analysed for human, mouse, rat and pig skin.

Materials and methods: Classic biophysical skin parameters were measured (e.g. pH, hydration, permittivity, transepidermal water loss). Physiological characteristics of the skins were also analysed by confocal Raman spectroscopy, scanning electron microscopy and two-photon microscopy.

Results: Based on biophysical testing, skin barrier function was damaged in psoriatic mouse skin and in marketed pig skin. Hydration and pH values were similar among the species, but freezing and thawing reduced the water content of the skins and shifted the surface pH to acidic. Aging reduced hydration and permittivity, resulting in impaired barrier function. Mechanical sensitization used in permeability studies resulted in proportional thinning of dead epidermis.

Discussion: Results indicate that depending on the scientific question it should be considered whether fresh or frozen tissue is used, and for certain purposes rodent skins are well usable. The structure of the skin tissue (ceramide, cholesterol, keratin, natural moisturizing factor or urea) is similar in rats and mice, but due to the higher skin thickness the lipid distribution is different in porcine skin. Psoriasis led to irregular chemical composition of the skin.

Conclusion: A comprehensive evaluation of skin samples of four species was performed. The biophysical and microscopic observations should be considered when selecting drug penetration models and experimental conditions.

KEYWORDS
aging, confocal Raman spectroscopy, dermal barrier, excised skins, freezing/thawing, hydration, scanning electron microscopy, tape stripping, TEWL, two-photon microscopy

1 | INTRODUCTION

In topical drug development and also in pharmacological, dermatological and safety studies it is important to investigate the penetration, pharmacokinetic/pharmacodynamic (PK/PD) profile and disposition of the transdermal drug formulations through and within the skin. There are in vitro models (such as artificial membranes with lipophilic or non-lipophilic characters), cell-based skin cell monolayers (such as HaCat cells)[1] and commercially available human reconstructed epidermis or full thickness living skin tissue models. These in vitro models have several advantages (stability, good reproducibility, no ethical issues), but they cannot mimic properly the full cellular composition of the living skin tissue. For this reason, excised fresh human and animal tissues are recommended for utilization in drug permeability or functional dermal barrier studies.

Earlier studies provided evidence that frozen and thawed excised skin tissues are not comparable with freshly prepared ones, and not usable for all purposes. For example, the efflux transporters, like P-glycoprotein, are destroyed during the freezing process, and the efflux pumps do not function properly after freezing and thawing.[2] The use of pig ears as a skin model of animal origin is common.[3–6] Also, rodent skin is frequently used in diffusion cells and drug release testing.[5,7–9] Previous studies of our research group focussed on measuring the effect of aging and application of different testing platforms (Franz diffusion cells, skin-on-a-chip microfluidic device, in vivo transdermal microdialysis) on the characteristics of topical drug absorption across the dermal barrier.[2,7,10] The animal skins of various species had different permeabilities for a hydrophilic model drug (caffeine) and for P-glycoprotein substrates (e.g., quinidine and erythromycin).[2,5]

To study in vivo skin diffusion, a novel non-invasive method is confocal Raman spectroscopy (CRS)[3,11–14] which has recently been developed and is suitable for testing skin composition and also epidermal penetration of different molecules at a specific time point. On the other hand the traditional transdermal microdialysis technique (another in vivo method) can be applied for continuously monitoring the transcutaneous absorption of the molecules across the full thickness skin or through the epidermis and dermis[5,15] (depending on the position of the microdialysis probes. The Raman technology is also used for ex vivo skins or in vitro skin substituents in several laboratories.[3,4,16,17]

The aim of the current study was to gain more knowledge about the physiological properties of excised animal tissues (mouse, rat, pig) and making a comparison with human skin. We also wanted to see the difference between the young and aged tissues with regard to their composition, in the easily available rat skin samples. To achieve our goals we performed a comparative analysis of (1) frozen and fresh skin samples, (2) young and aged skin samples, (3) abdominal skins from different species, (4) pig skins from a local market and from a veterinary farm; in addition, (5) the gradual effect of a routinely used mechanical skin pretreatment method (adhesive tape stripping) on the morphological characteristics of the skin surface was tested. To answer our questions, classic biophysical skin parameters were measured,[18] such as skin hydration (Corneometer, Epsilon sensor), pH of the stratum corneum surface, transepidermal water loss (TEWL, by Aquaflux) to assess dermal barrier function. To characterize the skin composition, 6 important components (ceramide, keratin, cholesterol, natural moistorizing factor, urea and water) were observed in the skins using CRS. For optical analysis, microscopic imaging (scanning electron microscopy [SEM] and two-photon microscopy) were performed.

2 | MATERIALS AND METHODS

2.1 | Excised skin samples

The animals used as skin sources served as non-treated controls in other experiments. Thus, intact skin physiology was ensured. At the end of the respective investigations, the animals were sacrificed by chloral-hydrate overdosing (i.p.). The hair was cut with an electronic trimmer and then the skin surface was treated with commercially available epilatory creams (X-Epil, Alveola Ltd. Budapest, Hungary) to facilitate analysis. The skin was exposed to the cream for 6 min. After removal of the epilatory cream, the skin was gently washed with tap water and wiped dry. A 20 mm diameter disc was marked on the smooth and dry skin and excised with scissors. Then one part of these samples was wrapped in aluminium foil and frozen at −80°C, while the other part was kept at refridgerated conditions (4–8°C) and freshly measured within 6 hours. The frozen skin samples were thawed on the day of the experiments at room temperature for 60 min. NMRI male mice (30–32 g), Wistar male rats (young: 280–330 g, old: 550–680 g), and young male pigs (15 kg) were used for the measurements. Some pig samples were provided by the University of Veterinary Medicine (Budapest, Hungary) while others were purchased from the Big Market Hall of Budapest. These last pig samples showed very different behaviour, presumably due to treatment during slaughtering as detailed in the results section. The human skin samples were purchased from a plastic surgery clinic (Révész Plasztika, Budapest) and used just as frozen samples. The donors were 40–42 years old females. The experiments were performed based on the permission for experimental application of human tissue No. 6501- 6 /2019/EKU, Budapest, Hungary. The excised human abdominal skin samples were cleaned from subcutaneous adipose tissue and stored at −80°C deep freezer, similarly to the animal tissue.

2.2 | Biophysical testing

2.2.1 | Skin hydration by Corneometer

Skin hydration is an important feature of skin samples used in experiments. Skin hydration measurements were conducted using a Corneometer CM825 mounted on a combination Derma unit SSC3 (Courage + Khazaka electronic GmbH, Cologne, Germany). This commonly used measurement probe is based on measurement of the electrical capacitance of the skin surface (uppermost 20 μm). Thus, the stratum corneum hydration is indirectly determined and obtained on an arbitrary scale, as the electrical capacitance depends on the skin's water content.[19] Measurements were performed in triplicate to obtain mean values ± SD.

2.2.2 | Skin hydration by Epsilon

The fingerprint sensor Epsilon (Biox Ltd., London, UK) is a permittivity-based measurement probe.[20] By means of capacitive contact imaging it can be used to determine the skin permittivity profile of skin samples on a larger area in real time, using an array of 76,800 capacitive sensors (12.8 x 15 mm area, 50 μm image resolution), which delivers results in numerical arbitrary units and 2D images. Dielectric permittivity ('dielectric constant') characterises the interaction of the investigated skin with an electric field.[21] The Epsilon sensor responds to capacitance, which depends on dielectric permittivity. The electrical capacitance of the skin is determined and thus information about the water content is obtained. While the Corneometer uses this princicple for a single sensor measurement, the Epsilon sensor was used to visually assess skin permittivity and at the same time observe the intactness of the skin samples, furrows and wrinkles. The skin relief can thus be taken into account with this device, more average values for skin hydration are obtained and areas with poor contact can be excluded. Moreover, the device offers a linearized calibrated response, unlike conventional corneometers.[21] Areas with no electric permittivity such as furrows are illustrated in black, while bright areas indicate high permittivity and therefore high skin hydration.[18] Measurements were performed in triplicate.

2.2.3 | Skin surface pH

While pH within the skin is not accessible in a non-invasive manner, skin surface pH can be determined by analysis of the hydrolipid film adherent to the stratum corneum. For skin pH measurements, the Skin-pH-Meter PH 905 mounted on a combination Derma unit SSC3 (Courage + Khazaka electronic GmbH, Cologne, Germany) was used. Following EEMCO guidelines, the electrode was calibrated regularly and samples were given time to acclimatize.[22] Measurements were performed in triplicate to obtain mean values ± SD.

2.2.4 | TEWL

The TEWL is an important parameter that is representative of the skin's barrier function in vivo. In ex vivo experiments, it can serve as an indicator for the integrity of the skin barrier function. TEWL measurements were performed with the condenser-chamber device AquaFlux AF200 (Biox Ltd., London, UK). With its closed-chamber principle the AquaFlux creates a microclimate within the measurement compartment. Thus, measurements remain unaffected by external air turbulences. The device measures water evaporating from the skin in g/m^2/h. Increased TEWL indicates skin barrier damage.[23–25] Measurements were performed in triplicate to obtain mean values ± SD.

2.2.5 | CRS

Confocal Raman Spectroscopy was performed for analysis of skin spectra, ceramide, keratin, cholesterol, natural moisturizing factor (NMF), urea and water content. Experiments were performed using a confocal Raman microspectrometer (gen2 Skin composition Analyzer, River Diagnostics, Rotterdam, The Netherlands) with two incorporated lasers, operating at different wavelengths (785 nm for analysis of the skin fingerprint region (400−1800 cm^{-1}) and 671 nm for analysis of the high wave number region (2500−4000 cm^{-1}). The device is regularly used for in vivo and ex vivo analysis of both human and porcine skin[3,16] and is able to monitor treatment-induced changes in skin parameters.

2.3 | Imaging techniques

2.3.1 | SEM

Samples where fixed and dehydrated for analysis through SEM. Fresh skin samples where immersed in 10% v/v formalin overnight and subsequently gradually dehydrated using a series of ethanol solutions as follows: 30% ethanol 2 h, 50% ethanol 2 h, 70% ethanol 2 h, 100% ethanol overnight (all % v/v). Before imaging, samples where removed from the solution, air dried, fixed on an aluminum sample holder stubs using double sided adhesive carbon tape and finally the remaining solvent was removed in vacuum for 10 min. Images were acquired using a Hitachi TM4000Plus II SEM using a 15 keV acceleration voltage in secondary electron mode (SE) at a 6 mm working distance.

Rat, mouse and human abdominal skins were studied with SEM. One group was sensitized by adhesive tape strippings (10 or 20 times); the other group was native without mechanical sensitization (no tape strippings).

2.3.2 | Two photon microscopy

Two-photon microscopic images were taken using a laser scanning two-photon microscope (Femto2D-galvo, Femtonics). The microscope

contained a 20× water immersion objective lens with 1.00 NA and 2 mm WD (XLUMPlanFL N, Olympus). The samples were excited between 830 and 920 nm wavelength with a femtosecond-pulsed two-photon laser (Chameleon, Coherent) and the fluorescence was collected using two GaAsP photomultipliers (PMTs) for green and blue detection (H11706P-40, Hamamatsu). The figures (Figures 7 and 8) were made with a MATLAB-based program (MES, Femtonics).

2.4 | Statistical analysis

Data are expressed as mean ± standard deviation (SD). Statistical comparisons were performed using unpaired student's t-test (MS Excel) or ANOVA (Graph Pad Prism); p-values < 0.05 were considered statistically significant. For non-parametric data, the Kruskal-Wallis test (Graph Pad Prism) was performed.

3 | RESULTS

3.1 | Biophysical testing

Skin hydration by Corneometer and Epsilon, Skin surface pH and Transepidermal Water Loss (TEWL) as indicators for dermal barrier function

In the first series of experiments, frozen skin samples from four different species (human, rat, mouse, pig) were compared in regard to their biophysical properties, including hydration, permittivity, pH and TEWL. The results are presented in Figure 1. All skin samples had abdominal origin and were not subjected to any mechanical pretreatment (zero tape strippings). The human, rat and mouse samples were native skins that had been excised and then frozen at −80°C in a freezer for a maximum of 3 months. Then the skin samples were thawed at room temperature and subjected to the measurements. The fourth species used was the pig. These skin samples were purchased from a local market, and most probably were subjected to sanitary surface treatment such as heating and scorching. This treatment had a significant effect on skin barrier function, as can be seen on the drastically increased TEWL (6–8 fold increase compared to the other species) (Figure 1A). Regarding the other parameters, the degree of skin hydration was highest in case of mouse skin (Figure 1C,D), while pH was highest for porcine skin surface (around 8.0).

In the second series of experiments, skin samples of young and old animals were compared for rat abdominal origin. With these experiments the effects of aging on the skin composition and also on the barrier function were tested. These data can provide information for the human skin aging. The samples were mechanically pretreated (10 tape strippings) as is necessary to perform effective permeability testing (Figure 2). The young and aged skin samples differed strongly in hydration. The water content of aged skins was significantly reduced (Figure 2C,D) compared to the young ones.

In the third series of measurements, fresh and frozen rat abdominal skin samples were compared (non-sensitized samples) (Figure 3). As shown in Figure 3C,D, the water content decreased and in Figure 3B, the pH shifted to acidic direction after the freezing and thawing process.

In the last experiments, four freshly excised tissues were examined (no mechanical sensitization). Three species were used (mouse, rat and pig). All of them were laboratory animals, the pigs were purchased from the University of Veterinary Medicine. The samples were epilated, except for a pig skin sample which was only shaved by razor. For comparison, an epilated pig skin was also tested. A diseased mouse skin of a chemically induced psoriasis model[26–28] was investigated as well (5% Aldara treatment for 96 h), but this sample (contrary to the others) had been previously frozen and thawed (Figure 4).

The TEWL values were lowest in case of normal mouse skin and highest in psoriatic mouse skin, indicating the degradation of the barrier function during the inflammatory process of psoriasis (Figure 4A). The hydration (Figure 4C,D) was similar in case of all ex vivo animal tissues. The pH values were quite similar, but a slight effect of epilation on the skin pH can be observed (shift into basic direction) in pig skin.

3.2 | CRS

The results of the CRS analysis of the different skin samples are depicted in Figure 5A–F. Each parameter of interest is plotted against the analysed skin depth from 0 micron down to 28 micron. The results depict mean values taken from $n = 3$ skin samples for mouse, rat and porcine skin and mean values of $n = 3$ measurements taken from $n = 1$ representative sample for the murine psoriasis model. Results are given as means ±SD of a higher number of individual sub-runs to provide better accuracy.

3.2.1 | Ceramides

The ceramide level showed a strong variation depending on species, skin state and skin depth. Mouse skin exhibited the highest ceramide levels at skin surface, but only for healthy mouse skin. The psoriasis model exhibited far lower values at the skin surface, as can be seen in Figure 5A. Also, the curve progression was different for the psoriatic mouse model.

Rat skin and porcine skin also exhibited significantly lower ceramide values at the skin surface than healthy mouse skin ($P < 0.05$ respectively). Interestingly, porcine skin showed a different progression of the curve with increasing skin depth and, together with the psoriasis mouse model, showed higher ceramide levels in deeper skin depths than mouse and rat skin.

3.2.2 | Cholesterol

The cholesterol levels showed a similar curve progression for most samples except for intact mouse skin, which showed a decline from

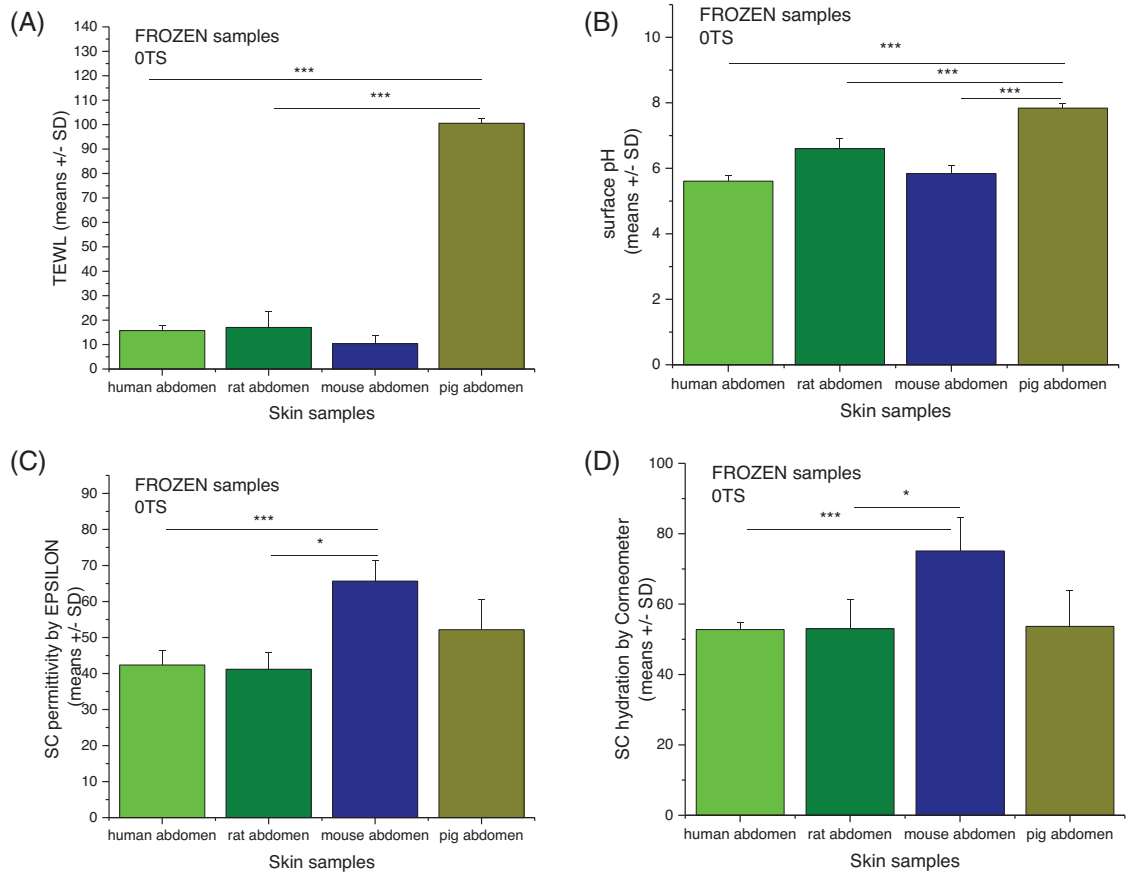

FIGURE 1 Comparison of biophysical parameters of excised, frozen native skin samples (0TS: no Tape Strippings, i.e., untreated) of four different species (human, rat, mouse, pig). (A) Transepidermal water loss (TEWL) representing barrier function, (B) stratum corneum surface pH, (C) permittivity by Epsilon sensor, (D) hydration by Corneometer. The number of experiments was $n = 5$/group. Pig skin was purchased from a local market, contrary to the other animal tissues which were collected from laboratory animals. Statistical significance is marked with $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.005$

the highest levels at the skin surface (Figure 5B). The reason for this deviation remains unclear and calls for further analyses.

3.2.3 | Keratin levels

Keratin levels were the highest not at the skin surface, but at 2–6 micron depth at the border of stratum corneum and living epidermis in all species tested, and the concentrations were different in healthy rodents contrary to diseased mouse and pig. For pig and psoriatic mouse skin the peak keratin concentration values were much higher than for healthy rodent skin (Figure 5C).

3.2.4 | NMF

The NMF values at the skin surface were highest for porcine skin, albeit with a large standard deviation (Figure 5D). Values showed a decrease with increasing meaurement depth, but remained higher than for the other species. This can be explained by the different structural composition of pig skin (according to the data of the literature the stratum coneum thickness for pig is approximately 12 micron, for mouse 2.9 micron and for rat about 5 micron.[29,30] Also the thickness of the epidermis is different: 52–100 micron for pig, 9.4–13.3 micron for mouse and approximately 21.7 micron for rats[29,30]. Mouse skin and rat skin showed far lower relative NMF values at the skin surface and in deeper levels, with a decrease after 4–6 micron of the stratum corneum. The psoriatic mouse skin showed lower NMF levels than intact mouse skin at the surface, but similar values at deeper measurement points.

3.2.5 | Urea

The urea levels (Figure 5E) showed low relative values at the skin surface for intact mouse and rat skin, with a curve progression of increase, followed by a decrease after 8 micron skin depth. Urea levels for pig skin again showed a different curve progression, which can again be explained similar to the differences in NMF levels. Likewise, the murine psoriasis model showed a completely different urea level at all measurement depths, indicating deviations from the normal physiological state.

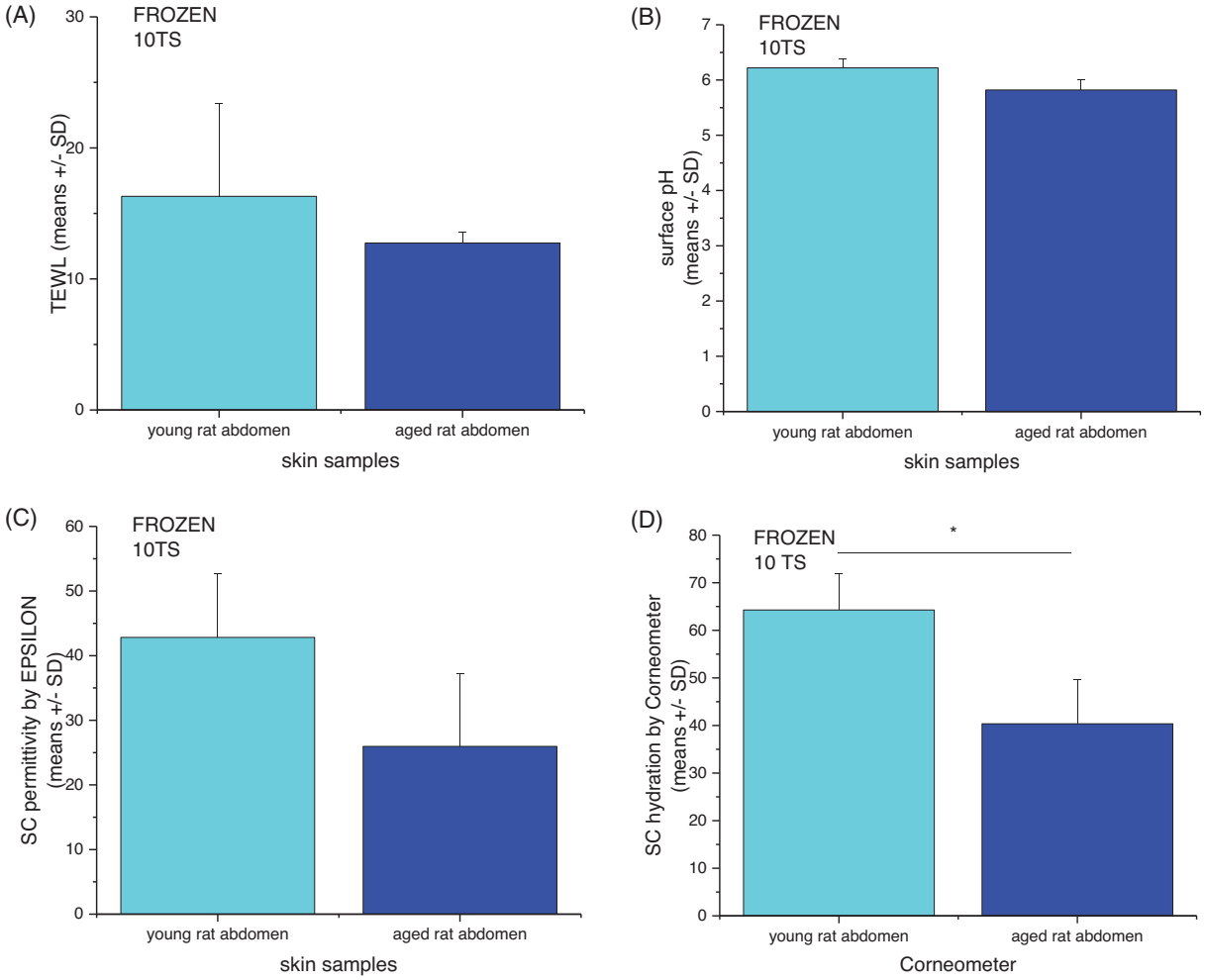

FIGURE 2 Comparison of biophysical parameters of excised, frozen and sensitized skin samples (10TS: 10 Tape Strippings) from young and old rats. (A) Transepidermal water loss (TEWL) representing barrier function, (B) stratum corneum surface pH, (C) permittivity by Epsilon sensor, (D) hydration by Corneometer. The number of experiments was $n = 5$/group. Statistical significance is marked with $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.005$

3.2.6 | Water content

The water content of the skin samples was highest for intact mouse and rat skin, which showed similar values and curve progression (Figure 5F). Again, porcine skin and the psoriatic mouse model were different in terms of total water content – they showed lower values, i.e. drier skin, and a more irregular curve progression. The total stratum corneum depth as derived from the water mass profiles was between 10 and 18 micron for all samples ($P > 0.05$).

3.3 | Imaging techniques

3.3.1 | Scanning electronmicroscopy

For topical drug diffusion studies mechanically sensitized excised skin samples (human or animal) are usually used as ex vivo diffusion platforms. The effect of tape strippings as a practical tool to increase the skin permeability has been widely studied. In the current SEM pictures it is clearly seen that 20 tape strippings removed the dead cell layers of the corneocytes (Figure 6) from the rat skin surface. Non-sensitized (0 TS) and 10–20 times sensitized rat skins were investigated. As can be seen in Figure 6, the 20 tape strippings practically removed the dead epidermis, and the appendageal elements became well-visible in the picture (hair follicles, sweat ducts, capillaries).

When comparing human and mouse abdominal skin (Figure 7) the density of hair follicles shows a very different pattern. In human skin, the number of hair follicles is about 11/cm^2, while in mouse skin this value is about 658/cm^2.[29] These structural properties have an impact on the appearance (wrinkles and creases) of the hairless skin surface as it is shown in Figure 7.

3.3.2 | Two-Photon microscopy

The effect of mechanical pretreatment is also well-visible with two-photon microscopic pictures (Figure 8). After the removal of ten adhesive tapes (10TS), the structure of the corneocyte layer on the skin

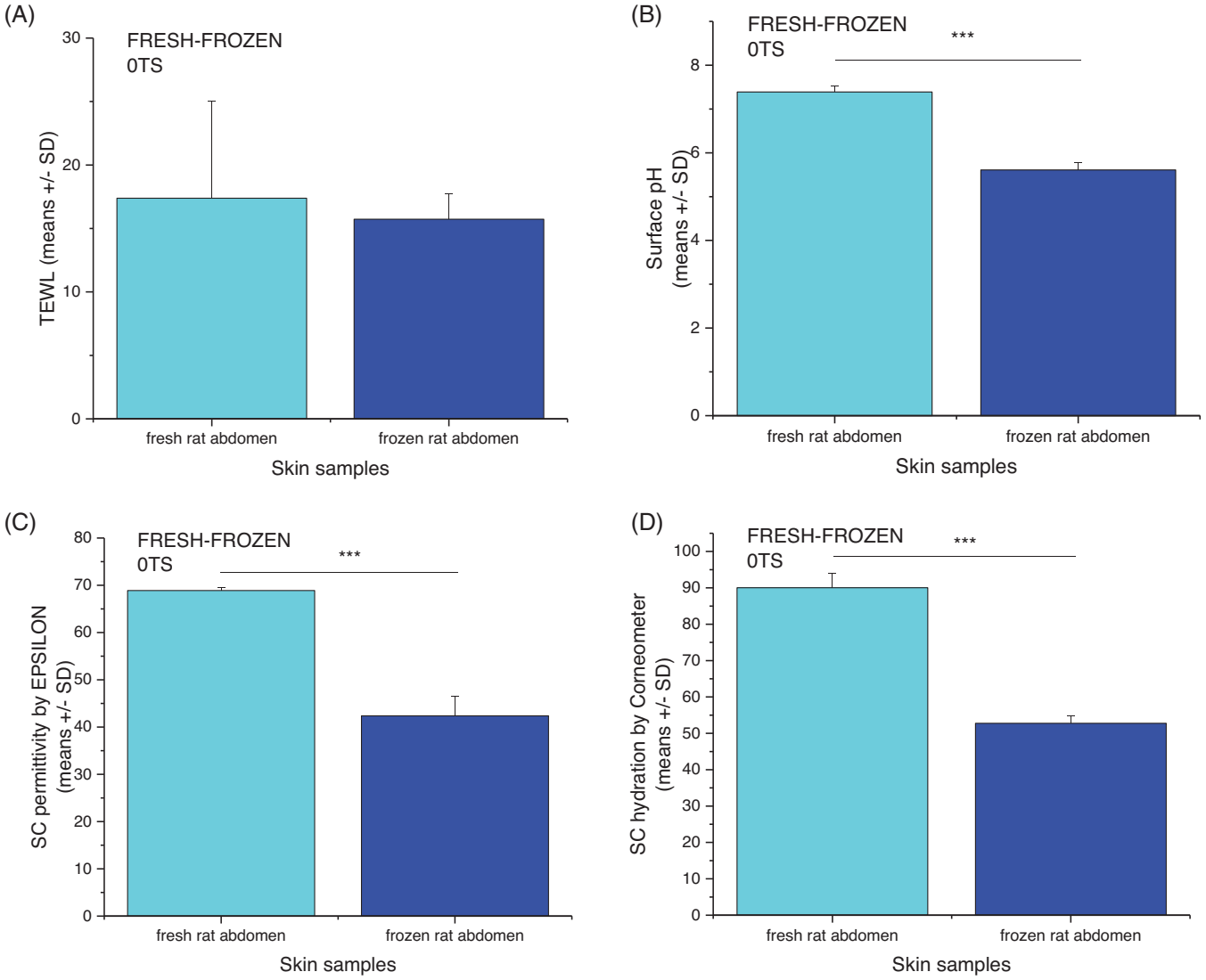

FIGURE 3 Comparison of biophysical parameters of excised, fresh, and frozen native (0TS: no Tape Strippings, i.e., untreated) skin samples from rat abdomen. (A) Transepidermal water loss (TEWL) representing barrier function, (B) stratum corneum surface pH, (C) permittivity by Epsilon sensor, (D) hydration by Corneometer. The number of experiments was $n = 5$/group. Statistical significance is marked with $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.005$

surface is changing. The connection between the cells becomes looser, the layer becomes less compact and therefore, the paracellular drug permeability can increase across the dermal barrier.

The cross sectional view of mouse skin is shown in Figure 9A. The green and blue autofluorescence can be seen in the surface corneocytes (pointed by red arrow in the figure) and in the subcutaneous adipocytes (lipid reach layer) (pointed by blue arrow in the figure), respectively. The surface view of the human abdominal skin cells (Figure 9C) is similar to the rat skin images shown in Figure 8.

4 | DISCUSSION AND CONCLUSION

Biophysical, physiological and morphological characterization of different excised skin samples were performed in this study. Pig and rodent skins are frequently used in topical drug delivery, safety and efficacy assays. In other cases, human skin samples are also available e.g. from plastic surgery clinics or from cadavers. However, the timing and the harmonization of the skin collection and the permeability experiments is not always easily feasible. It requires strict organization and even so, skin is not always available due to short-term changes and irregularities with patients or animals. Therefore, in many cases skin tissues are stored frozen until the experiments, but storage conditions may have an influence on the test results.[31] It was described that in specific studies (e.g. testing the efflux transporter functionality in the skin) such frozen samples cannot be utilized.[2] In the current study, some remarkable biophysical differences were shown between fresh and frozen tissues (surface pH, hydration, permittivity). Also at observation of young and aged skin samples, characteristic differences were seen in the water content and electrical conductivity at the skin

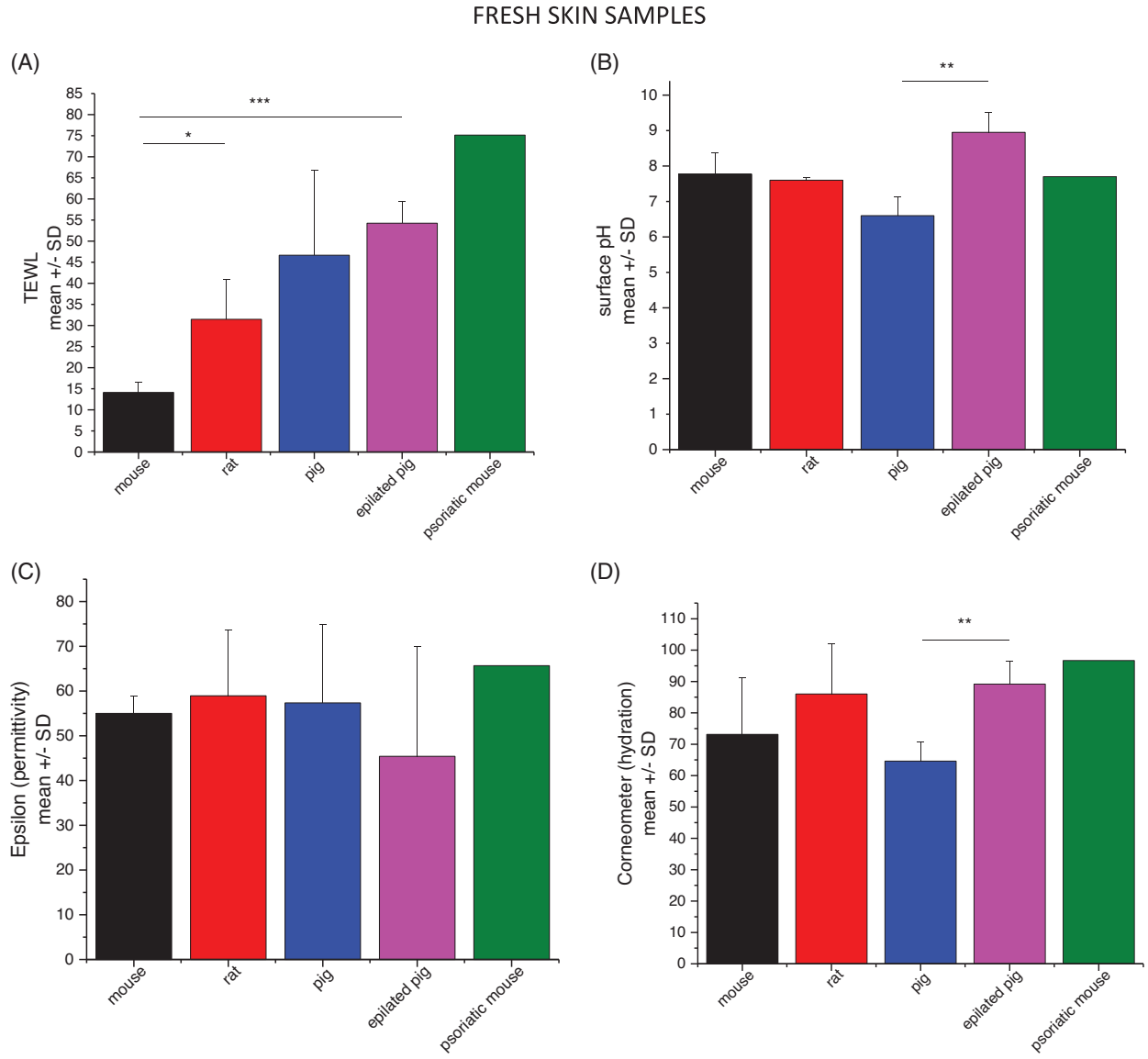

FIGURE 4 Comparison of biophysical parameters of excised, fresh native skin samples (0TS: no Tape Strippings, i.e., untreated) from different species. (A) Transepidermal water loss (TEWL) representing barrier function, (B) stratum corneum surface pH, (C) permittivity by Epsilon sensor and (D) hydration by Corneometer. The animal skins (abdominal) were measured ex vivo, while the human skin was tested in vivo on the inner forearm as a control. the number of experiments was $n = 3$/group of mice, rats and pigs and $n = 1$ for psoriatic mouse skin and in vivo human control. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.005$

surface. These results are in accordance with the results of other research groups.[32,33,34]

When comparing the skin properties of different species the results revealed that pig skin samples purchased from a market cannot be used for pharmacological or pharmaceutical experimental purposes because of the inherent cleaning treatment after sacrifice of the animal. This process resulted in a complete disruption of the dermal barrier as it was shown in the current study. In the pilot study with a psoriatic mouse skin sample, huge deviations were observed compared to intact mouse skin with regard to the barrier function (TEWL).

Analysing the physiological components of the skin, ceramide, cholesterol, keratin, NMF, urea and water contents were tested in this study at different skin depths by CRS. The human stratum corneum includes 50% ceramides, 25% cholesterol and 15% free fatty acids.[35–37] Ceramides are a family of waxy lipids. They are present in high concentration in the cell membrane as components of the lipid bilayer. Their physiological role, together with cholesterol in the skin, is that they are the main components of stratum corneum in the dead epidermis and create a water-impermeable barrier which is protective for the organ and prevents excessive water loss. Ceramides also create a barrier against skin surface microorganisms.[38,39] Keratin is one of a family of fibrous structural proteins known as scleroproteins. α-Keratin is a type of keratin found in vertebrates. It is the key structural material making up scales, hair, nails, feathers, horns, claws, hooves, calluses, and the outer layer of skin among vertebrates.[40] NMF and urea are also important elements of the upper skin. Urea, a component of

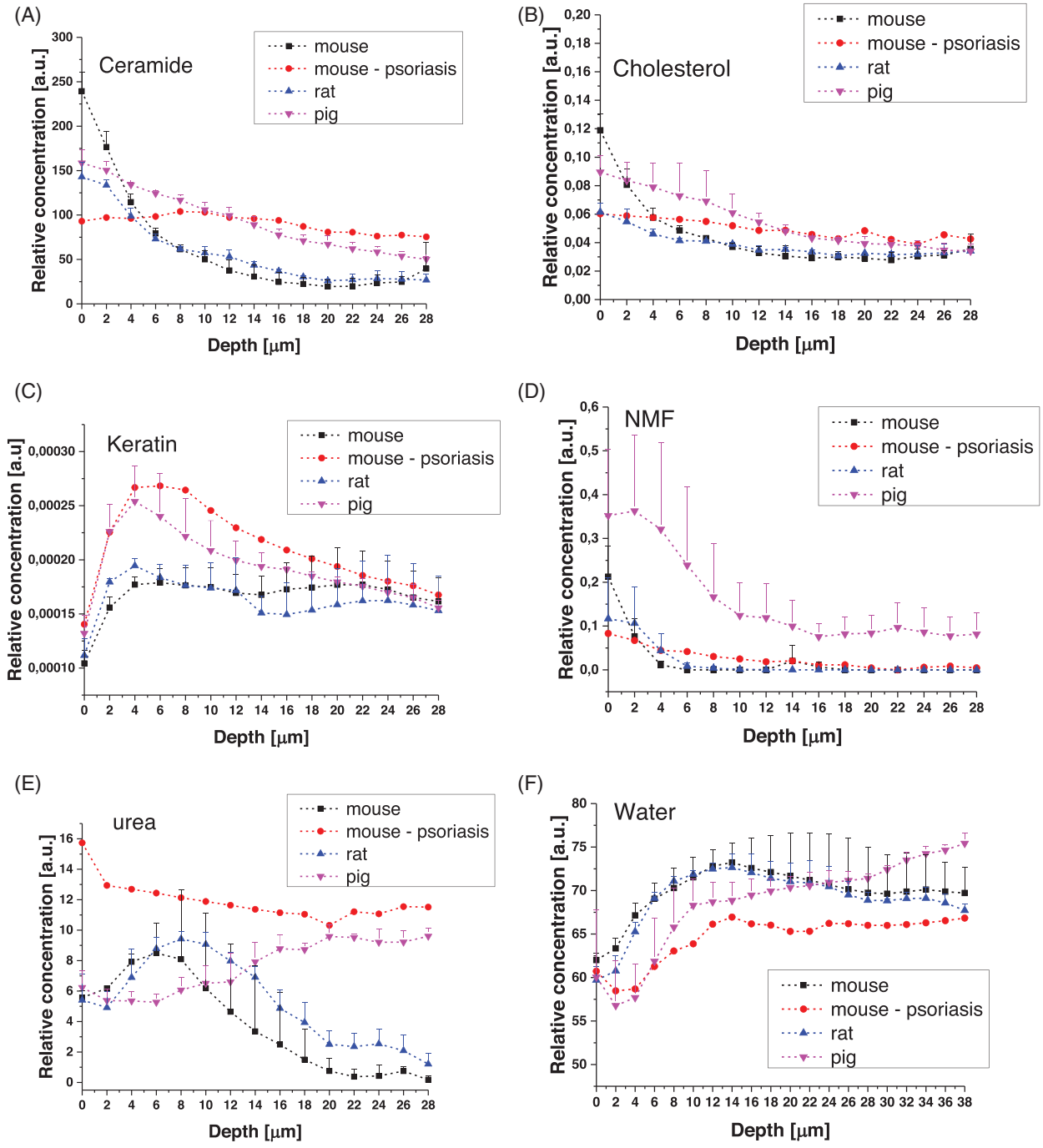

FIGURE 5 Confocal Raman spectroscopy (CRS) depth profiles of physiological skin parameters measured in normal and diseased (psoriatic) fresh mouse skins, rat, and laboratory pig skins. (A) Ceramide content, (B) cholesterol content, (C) keratin content, (D) natural moisturizing factor content, (E) urea content, (F) water content of the skin at different measurement depths from skin surface (0 micron) and stratum corneum until the dermis (28 micron). The number of experiments was $n = 3$ samples/group except for psoriatic mouse skin ($n = 1$ sample); all measurements were performed with individual measurements of $n = 5$–8 in the fingerprint region and $n = 4$–6 in the HVN region; results are given as means +/- SD

the NMF of the skin, plays an important role in the preservation of skin hydration and integrity. At lower doses (≤10%), urea is a skin moisturizer, while at higher concentrations (> 10% urea), it exerts a keratolytic action. Urea is also useful in combination therapies with anti-inflammatory and anti-fungal drugs due to its activity as a penetration enhancer.[41]

Hydration is a key aspect of the skin that influences its physical and mechanical properties. The upper layer of the skin generally has the lowest water activity, while the deeper layers are close to physiological water activity.[42] The hydration of the skin is changing during aging. Skin aging is a multifactorial process resulting from intrinsic and extrinsic factors. Intrinsic factors are associated with the influences

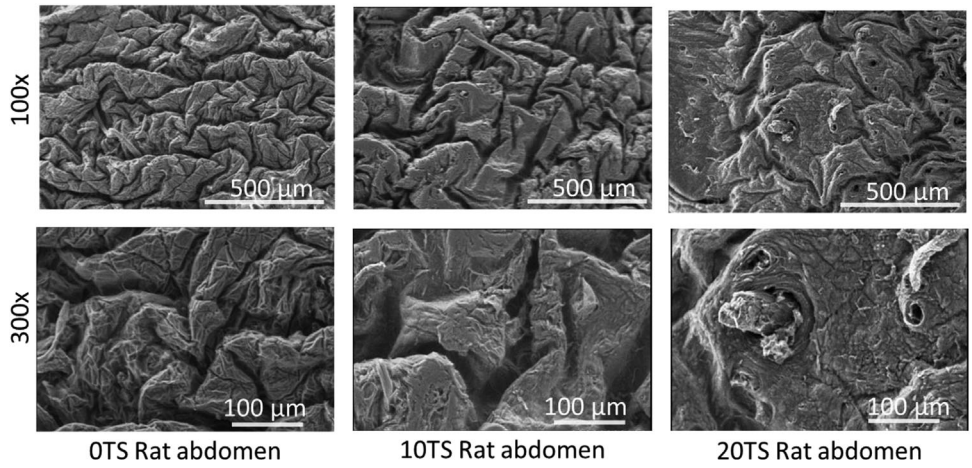

FIGURE 6 Comparative scanning electron microscopy to study the effect of tape stripping on skin surface morphology. Rat abdominal skin after shaving and epilation without mechanical sensitization (0TS), with 10x (10TS) and 20x sensitizations (20TS)

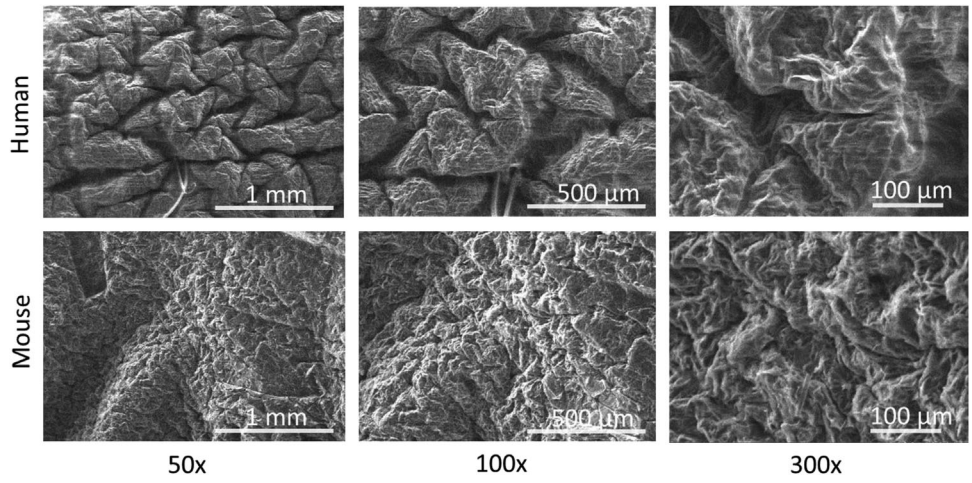

FIGURE 7 Comparative scanning electron microscopy of the skin surface of two species. Upper panels: human abdominal skin, lower panels: mouse abdominal skin after shaving and epilation, all without mechanical sensitization (0TS)

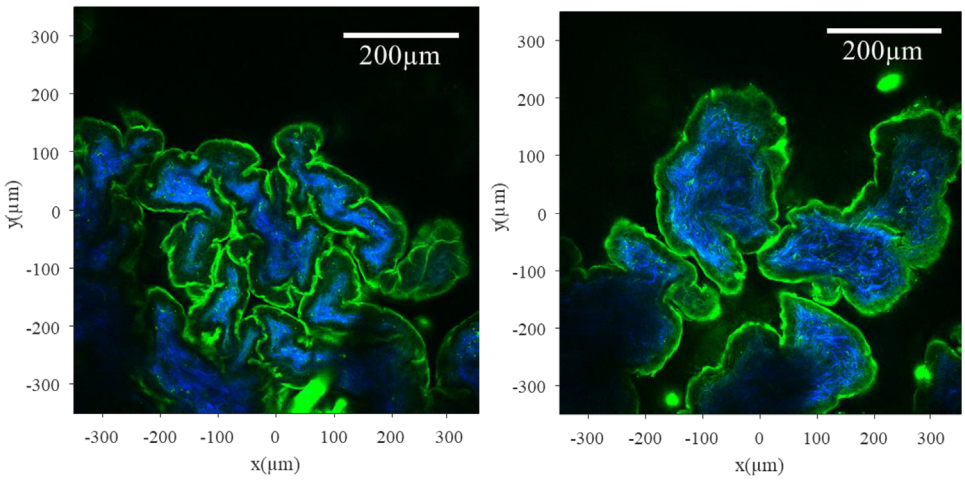

FIGURE 8 Two-photon microscopy of excised rat abdominal skin. Left: intact skin without mechanical sensitization (0TS), Right: with 10x mechanical sensitizations (10TS). The skin samples were not labelled, they show autofluorescence at the given laser wavelengths

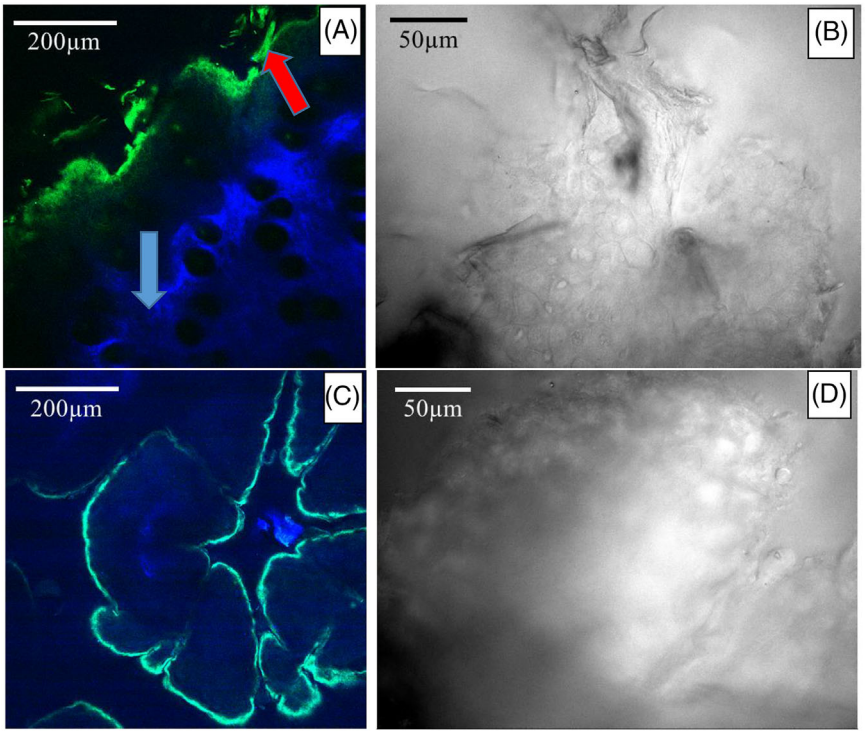

FIGURE 9 Two-photon microscopy of mouse (A and B) and human (C and D) abdominal skin samples. Left: laser pictures, right: camera pictures. 'A' is a cross sectional view, 'C' is an air interface surface view. Red arrow indicates the skin surface stratum corneum. Blue arrow indicates the subcutaneous adipocytes

of genetic, hormonal, and metabolic slowdown, whereas extrinsic factors include the exposure to solar radiation, pollutants, and lifestyle behaviors.[43] Both factors influence the changes in skin appearances, including skin dryness, laxity, dynamic and static wrinkles, and irregular pigmentation.[44] To further examine the process of skin aging in vivo Raman spectroscopic studies can be proposed.

Our multifactorial analysis of the skin components as function of skin depth revealed that mice and rats have very similar skin chemical composition, while that of the pig skin was different (higher NMF and keratin content and lower water content in the surface, but higher in the deeper epidermal layers). Comparing the intact and the psoriatic mouse skins all physiological parameters were different - indicating an irregular pathological status of the psoriatic model.

When comparing the Raman data of the animal skins with human skin in vivo,[45] the NMF and urea distribution throughout the stratum corneum were most similar in case of porcine skin. For ceramides, higher relative concentrations were observed with the animal skins; this might be an artifact caused by residues of external products such as the depilatory cream in this study. Also, higher relative water mass was observed for the animal skins and the threshold to the constant water mass of the living epidermis was reached much sooner. Thus, the estimated stratum corneum thickness can be expected to be lower for the animal skins in comparison to human skin.

No Raman measurements of human skin were included in this study due to the high legislative efforts.

Porcine samples were collected from the caudal abdominal area in the current study. In a previous article Khiao and co-workers reported[46] that comparing the porcine ear, flank, back and caudal abdominal skins with human abdominal skin samples from plastic surgery there were no significant differences between the two species and the various anatomical areas. Histological and ultrastructural assessments were carried out on the epidermis and dermis, focusing on the dermo-epidermal interface length, dermo-epidermal thickness ratio as well as densities of hair follicles, arrector pili muscles, blood vessels and sweat glands. Results showed that both histologically and ultrastructurally, all four regions of porcine skin were similar to human skin.[46]

Considering the different data sets, some conclusions on the use of skin models can be made. Skin samples of aged animals may exhibit reduced hydration (Figure 2). The use of frozen skin samples is often necessary from a practical viewpoint; one should however be aware that the freezing and thawing process may affect skin parameters such as hydration (Figure 3) and should be well standardized. Pre-treatment by hygiene procedures during processing of industrially used animals should be avoided since it leads to elevated TEWL and lower barrier function (Figure 1). Inter-individual variability between samples needs to be taken into account for all species and all setups. In total, mouse and rat skin exhibited similar properties in many of the experiments (e.g. when considering the Raman depth profiles for keratin, NMF, urea and water).

The experiments on fresh skin samples showed differences in TEWL, i.e. barrier function, which are consistent with current knowledge on lower barrier function in rodents (thinner stratum corneum) and pig skin (different lipid matrix composition).[16] In total, however, the strong variability led to overall comparable values especially for pH and hydration.

The morphological analysis of the sensitized and intact skin samples revealed the thinning of the corneocyte layers (10 TS) and also the almost complete removal of the epidermis after 20 tape strippings.

The appendages (sweet ducts, capillaries, hair follicles) were exposed in this preparation. The two-photon microscopic pictures showed the disintegration of the surface structure of the dermal barrier after tape removal, and in the cross sectional view the subcutaneous fatty tissue became also visible.

In conclusion, a multiparametric comparison of ex vivo skin samples of four different species was performed. Based on the results, the mouse and rat skins showed similar biophysical characteristics and chemical composition with a very low variability. These parameters greatly changed in chronic inflammatory conditions in psoriatic mouse skin. The structural ingredients were different between the rodents and pig skins. The freezing and thawing process had a remarkable effect on biophysical parameters, and aging greatly reduced the hydration level and permittivity of the skins. The frequently applied mechanical pretreatment procedure was evaluated by SEM and two-photon microscopy. Both techniques showed that removal of 20 TS is not appropriate for performing relevant drug delivery studies with rat skin for dermal preparations because it results in almost complete lack of the entire epidermis. Our findings indicate that before selection of a skin model and storage conditions it is very important to review literature data about the characteristics of the skins and to consider if the species, the tissue preparation and the assay conditions are appropriate and applicable to answer our scientific questions. From the presented experiments and previous studies, it can be derived that fresh and untreated skin samples are preferable. If human skin is not an option, untreated porcine skin and rodent skin can both be used. Furthermore: (1) for permeabilty studies both fresh and frozen tissues can be used, (2) for functional studies e.g. on transmembrane transporters, only fresh tissues are recommended (Bajza et al, 2020), (3) for dermal irritation and inflammation markers frozen tissues can also be applied, (4) for cosmetic testing pig skins are preferable contrary to rodent skins (density of hair follicles, thickness of the stratum corneum, NMF, urea, water content and elasticity are more similar to human tissue).

ACKNOWLEDGEMENTS

The psoriatic mouse skin provided by Prof. Rolland Gyulai and his group (University of Pécs) is gratefully acknowledged. The authors are also grateful to Ms. Erika Pári (Révész Plasztika, Budapest) for providing and preparation of human excised skin samples and Prof. Balázs Rózsa for two-photon microscopy. Project no. ED_17-1-2017-0009 has been implemented with the support provided by the National Research, Development, and Innovation Fund of Hungary, financed under the National Bionics Program funding scheme. This research was also supported by the National Research, Development and Innovation Office through the grant TKP2021-EGA-42.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

DATA AVAILABILITY STATEMENT

Data are available in the laboratory records and computer archives.

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How to cite this article: Kocsis D, Klang V, Schweiger E-M, et al. Characterization and ex vivo evaluation of excised skin samples as substitutes for human dermal barrier in pharmaceutical and dermatological studies. Skin Res Technol. 2022;28:664–676. https://doi.org/10.1111/srt.13165