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Research paper

TEWL measurements as a routine method for evaluating the integrity of epidermis sheets in static Franz type diffusion cells in vitro. Limitations shown by transport data testing

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Abstract

The suitability of transepidermal water loss (TEWL) measurements in vitro as a barrier integrity test for human heat separated epidermis (HSE) was investigated. A model system consisting of a Teflon membrane mounted in Franz diffusion cells (FDC) filled with phosphate buffer saline (PBS) was set up. The membrane was used intact and punctured with a needle (up to five holes). After each puncturing the TEWL was measured. Only the TEWL of intact and punctured membrane differed significantly regardless of the number of holes. From three donators intact human HSE and punctured HSE were compared and no significant difference of the TEWL was found. Permeation experiments with flufenamic acid (FFA) showed a significantly higher diffusion rate through punctured HSE. TEWL and drug permeation were compared for skin stripped three, seven and 15 times prior to heat separation to an intact control group. Only the TEWL values of intact HSE and HSE stripped 15 times differed significantly. However, seven and 15 times stripping resulted in significantly higher diffusion rate. In conclusion, TEWL measurements can detect severe damage of the stratum corneum (SC) but not small changes, which nevertheless may already influence drug diffusion. Therefore, TEWL measurements appears to be of limited use as a barrier integrity test for human HSE in in vitro test systems.

Keywords: Tewameter; TEWL; Barrier integrity test; Human skin; Heat separated epidermis; Permeation data

1. Introduction

The measurement of transepidermal water loss (TEWL) is a well established method in dermatology to assess the integrity of the skin barrier in vivo [1]. When skin is damaged, its barrier function is impaired resulting in higher water loss.

A frequent problem in the routine practice of diffusion experiments in Franz type diffusion cells (FDC) is the lack of suitable methods for routinely assessing the integrity of epidermal sheets inside the diffusion cell before the experiment has started. However, the OECD guideline 428 [2] requests integrity testing before performing permeation experiments and the Guidance document 28 [3] specifies this statement by recommending the measurement of TEWL, electrical resistance or the use of tritiated water as permeation marker. In comparison with the other methods, TEWL measurements have the advantage that no solutions have to be added to perform the barrier integrity test other than those used in the permeation experiments. Therefore, this investigation was focused on the potential of TEWL measurements to be used for routine testing for the integrity of human HSE prepared for in vitro permeation experiments in a Franz type diffusion cell. Furthermore, the influence of the degree of skin surface damage on drug permeation was addressed. For this purpose the stratum corneum (SC) barrier was damaged by punctuation and stripping with adhesive tape. TEWL measurements and permeation experiments were carried out using the same membrane in both experiments. As model drug flufenamic acid was chosen.

Abbreviations HSE, heat separated epidermis; FDC, Franz type diffusion cell; FFA, Flufenamic acid; PBS, Phosphate buffer solution; SC, Stratum corneum; TEWL, Transepidermal water loss.

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2. Materials and methods

2.1. Materials

The following chemicals and equipment were used: flufenamic acid (Sigma-Aldrich, Deisenhofen, Germany); phosphate buffer solution pH 7.4, McIlvaine citric acid-phosphate buffer pH 2.2 (all components from Merck, Darmstadt, Germany); methanol (VWR International, Leuven, Belgium); Teflon membrane (thickness 1 mm) (Arthur Krüger, Brasbüttel, Germany)

2.2. Human heat separated epidermis

Human skin from caucasian female patients, excised during abdominal plastic surgery, was used with the approval of the ethic commission of the Caritas hospital Lebach, Germany. The subcutaneous fatty tissue was removed immediately after excision. The cleaned skin was stored at −26 °C until use. Previous studies had shown that the skin is stable over 3 and 6 month [4]. This has been confirmed by other laboratories [5,6].

Skin discs with a diameter of 25 mm were punched out and thawed prior to putting them in water at 60 °C for 1 min. Afterwards the SC-epidermis layer of the skin was peeled off from the dermis using forceps [7]. The epidermis sheets were spread out in Petri dishes filled with PBS for at least 30 min.

2.3. Punctured human heat separated epidermis

Before heat separation the skin disks were punctured with a needle with a diameter of 1 mm in the center of the diffusion area in order to simulate damage sustained during surgical extraction or preparation. After heat separation the presence of the hole was checked with a magnifying glass.

2.4. Stripped human heat separated epidermis

Tape stripping has often been used to induce barrier disruption on human skin [8–10] and was therefore used in this investigation to inflict varying, but defined damage on the stratum corneum barrier. The skin was stripped prior to heat separation. The numbers of adhesive tapes used were fixed to three, seven and 15; the stripping procedure is described in detail by Wagner [11]. Stripping more than 15 times could not be performed due to the high fragility of the resulting HSE sheet.

2.5. Experimental design for TEWL measurements

For TEWL measurements a TEWAMETER Multi Probe Adapter 5 (Courage and Khazaka, Köln, Germany) was used and positioned in a cabinet drier set to 25 ± 1 °C. The FDC setup was transferred to the drier. Using a telescopic arm the TEWL sensor was placed into the opening of the FDC. Another sensor (ambient condition sensor, Courage and Khazaka, Köln, Germany), for humidity and temperature, was added, too. Both sensors were linked to a computer. During measurements the relative humidity (49 ± 5%) in the cabinet drier was subtracted constantly from the TEWL value. The drier was closed and the measurements were initiated when the temperature was stable at 25 ± 1 °C, usually 30 min later. The TEWL was then measured every 15 s over 7 min resulting in 28 data points.

2.6. Testing and characterization of the system via Teflon membranes

To examine whether an increasing damage of a membrane was reflected in a higher TEWL value, a Teflon membrane was placed in the FDC to act as an artificial diffusion barrier. To tightly seal the acceptor from the donor compartment, petrolatum was applied between the Teflon membrane and the upper and lower part of the FDC. Subsequently, the lower part of the FDC was filled with PBS. Preliminary experiments had shown that the distance between sensor and membrane surface had to be reduced to 3 mm. Due to this reason a cut off upper part of a FDC was used. The experiments were carried out as follows: Six FDCs were mounted with intact Teflon membranes and the first set of TEWL measurements was performed. Then the membranes were punctured with a needle and the TEWL was measured again. This procedure was repeated until a total of five holes was reached.

2.7. Experimental setup using human heat separated epidermis

The experimental setup had to be optimized to fulfill the special requirements necessary while working with human HSE. Preliminary experiments demonstrated that it took around 15 min for topically adhering water to evaporate (Fig. 1). To avoid this problem the experimental system was left without cover for 30 min in the cabinet drier. Topically adhering water could evaporate and an equilibrium was reached. The TEWL measurements were initiated afterwards.

To be able to use the same experimental setup firstly for TEWL measurement and secondly for permeation experiments

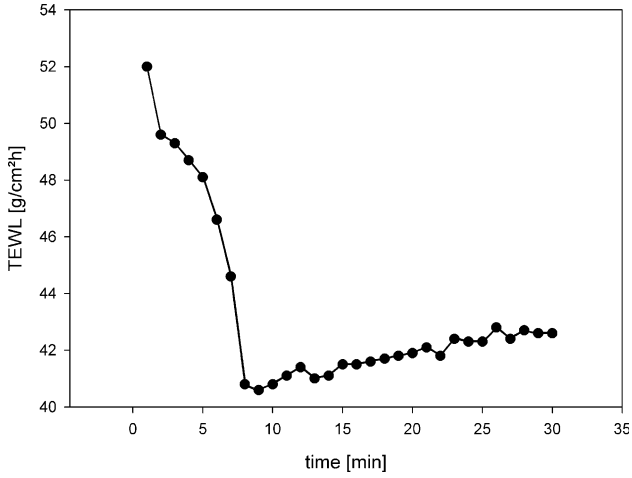

Fig. 1. TEWL values over time obtained from a not correctly dried HSE sheet.

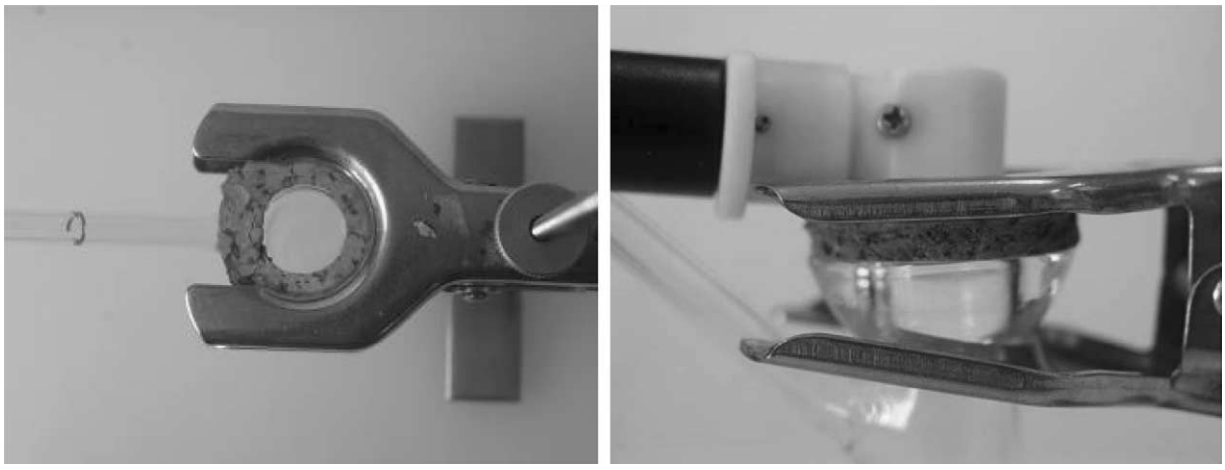

Fig. 2. Experimental setup of TEWL experiments.

the following procedure was adopted: The upper part of the FDC was replaced by a cork ring and a ring of a dialysis membrane. At the center of that dialysis membrane an opening with a diameter of 16 mm, slightly larger than the diffusion area in the permeation experiments, had been punched out. This membrane ring was placed on top of the HSE. The whole system was then secured with a horse shoe clamp to avoid accidental displacement. The cork ring and the clamp assured that the HSE was securely placed on top of the FDC. The cork ring kept the HSE from being damaged by the clamp. The dialysis membrane ring allowed the removal of the cork ring after the TEWL measurements were carried out without the HSE sticking to the cork ring. The opening at the center of the cork ring was large enough to place the sensor 3 mm above the surface of the epidermis membrane. The distance was controlled by using a micrometric ruler. The experimental setup is shown in Fig. 2.

2.8. Permeation experiments

For the following diffusion experiments the cork ring was replaced by the original upper part of the FDC. The same HSEs as in the TEWL experiments were used.

Permeation experiments were carried out in Permegear static type 6G-01-00-15-12 Franz cells (Perme Gear, Riegelsville, PA, USA); receptor volume: 12.1 ml; donor volume: 0.5 ml 1250 μg/ml of Flufenamic acid in PBS buffer; diffusion area: 1.768 cm^2; acceptor: PBS buffer. At defined time intervals 0.4 ml samples were drawn and replaced by fresh PBS. For further details see Wagner [12].

2.9. Calculation of Papp value

The linear branch of the permeation data was determined using correlation analysis. A minimum of six data points in the linear branch were taken to calculate the flux J [μg/(cm^2s)] by linear regression. The flux J was then divided by the concentration in the donor [μg/cm^3] in order to calculate the apparent permeation constant Papp [cm/s].

2.10. Drug analysis

From each sample 50 μl were injected directly onto an isocratic HPLC system consisting of a Dionex ASJ 100 automated sample injector, an UVD 170S detector, a Dionex P580 pump, Chromeleon 6.50 SP2 build 9.68 and a 5 μm LiChrospher® 100/RP-18 column/12.5 cm × 4 mm (Merck-Hitachi, Darmstadt, Germany). An 80/20 (V/V) mixture of methanol/McIlvaine citric acid–phosphate buffer pH 2.2 was used as a mobile phase. At a flow rate of 1.2 ml/min the retention time of FFA was 3.5 ± 0.2 min. The detector was set at 284 nm. Unknown FFA concentrations were calculated against known standards via the method of area under the absorption-time curves. The method provided good linearity ($r = 0.999$) over a concentration range of 50–2000 ng/ml.

2.11. Statistical evaluation

All statistical evaluations were done with Sigmastat 3.0.1. (SPSS Inc., Chicago, USA).

3. Results

3.1. Experiments with Teflon membrane

As shown in Fig. 3, the TEWAMETER could only distinguish between an intact and a punctured Teflon membrane. A significant increase of the TEWL value was observed after the first hole but did not rise any further adding more holes (Fig. 3).

3.2. Experiments with punctured epidermal sheets

Considering the results with the Teflon membrane six FDCs were prepared with intact human HSE and six FDCs with HSE sheets each punctured with one hole to compare their TEWL values. This experimental design was repeated with HSE sheets of two additional donors (Fig. 4). Independent of the skin used no significant differences could be found (t-test with

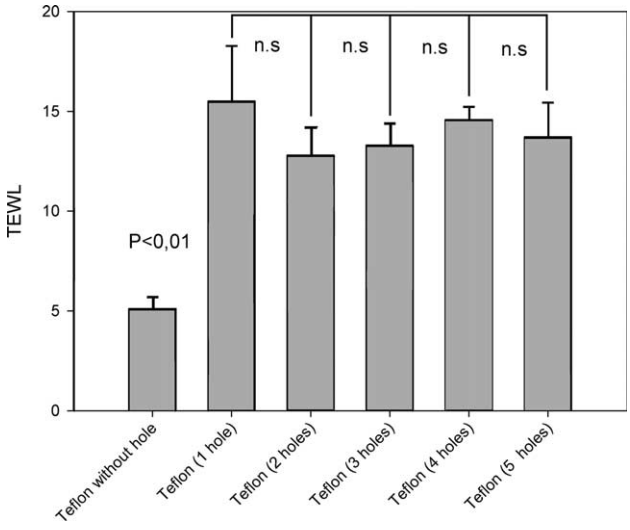

Fig. 3. Results of experiments with Teflon membranes (all groups $n=6$).

alpha = 0.05: set A: $P=0.075$; set B: $P=0.375$; set C: $P=0.282$). The TEWL measurement was not suitable to identify those small skin damages.

Furthermore, permeation experiments with FFA were carried out with set A, B and C to investigate the impact of the punctuation on drug diffusion (Fig. 5).

In each set, the Papp values of punctured HSE were compared with the intact HSE control group using a *t*-test (*t*-test with alpha = 0.05; set A: $P<0.01$; set B: $P<0.01$; set C: $P<0.01$). A statistically significant difference was found in all three data sets.

3.3. Experiments with stripped skin

In this experimental series, a control group with undamaged HSE ($n=6$) was compared with a stripped epidermis group consisting of HSE sheets stripped 3 ($n=6$), 7 ($n=6$) and 15 ($n=6$) times prior to heat separation. The TEWL values of all HSE membranes are shown in Fig. 6. A statistically significant difference was only found between intact HSE and HSE stripped 15 times (for statistical comparison ANOVA one way [13] was used [alpha = 0.05; $P<0.05$; null hypothesis = no difference between the experimental groups]). The Holm-Sidak method was taken to identify significantly differing groups ($P=0.05$). A statistically significant difference was only found between intact HSE and HSE stripped 15 times.

The results of the subsequent permeation experiments are shown in Fig. 7. The Papp values of all treatments were compared with Kruskal–Wallis Analysis of Variance on Ranks (null hypothesis = no difference in the distribution of values between the different groups). The Kruskal–Wallis ANOVA on Ranks is a non-parametric test that does not require assuming all the samples were drawn from normally distributed populations with equal variances [13]. The application of this test was necessary since one group of data was not normally distributed. The null hypothesis was rejected. Therefore, the groups demonstrated a significant difference ($P<0.05$). Now, a Holm-Sidak procedure could be used (overall significance level = 0.05) to find out which groups differed significantly. A statistically significant difference was determined between intact HSE, punctured HSE and HSE stripped seven and 15

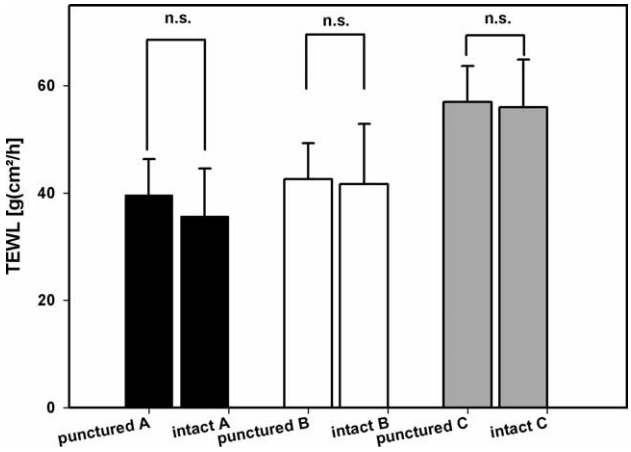

Fig. 4. TEWL values of punctured and intact HSE (all groups $n=6$).

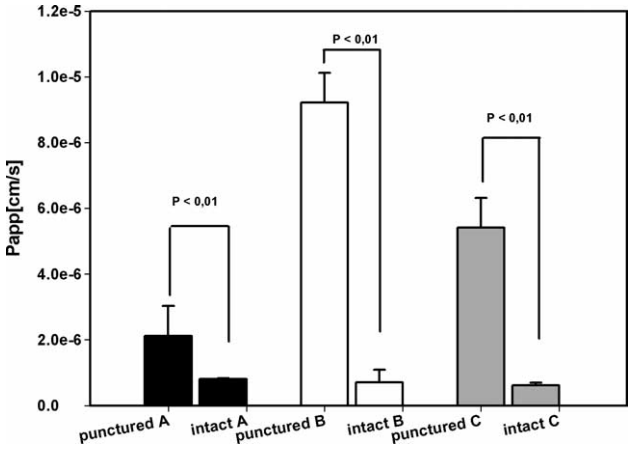

Fig. 5. Results of permeation experiments using intact and punctured HSE as barrier (all groups $n=6$).

times. No difference could be detected between intact HSE and HSE stripped only three times.

4. Discussion

The TEWL is a well established method for testing the integrity of the barrier function of the SC of human skin in vivo [14–20]. Therefore, this method is often recommended in the literature [3] as a barrier integrity test for in vitro permeation experiments.

The first experiments with the TEWAMETER showed that in order to be able to use TEWL measurements as a barrier integrity test in static FDCs the experimental parameters had to be adjusted. Two factors proved to be of crucial importance: First, reproducible data were only obtained when the sensor of the TEWAMETER was positioned 3 mm above the membrane. Second, the experiments had to be performed in a cabinet drier to avoid air turbulences and set at constant temperature (in our case 25 ± 1 °C). One method to place the sensor is shown in Fig. 2. Another difficulty encountered during this test phase was the water adhering on the HSE's surface. To overcome this problem an equilibrium time of at least 30 min was necessary (Fig. 1). When these factors were taken into account it became possible to distinguish intact Teflon membranes from punctured Teflon membranes as shown in Fig. 3. The lipophilic Teflon membrane was used in order to make sure that water could only cross the barrier at the puncture holes. Although only the influence of the holes on the TEWL should be addressed, it was not possible to differentiate between membranes with a different degree of damage, e.g. one to five holes. Since, water droplets could be seen on top of the holes while performing the experiments a possible explanation might be that this water covers the surface of the membrane with a thin water layer—independently of the number of holes.

Experiments with punctured HSE sheets (one hole) of three different donors showed no statistical significant difference between damaged and intact HSE concerning the TEWL value (Fig. 4). One explanation might be, that due to swelling processes, the holes were sealed [3]. Similar findings had been reported for holes in guinea pig skin resulting from the hairs [21]. In contrast to the TEWL results, permeation experiments with punctured HSE demonstrated a strong influence on drug diffusion, which show that the integrity of the barrier was compromised. As indicated in Fig. 5 the Papp value for the damaged HSE sheets is 2–10 fold higher compared to the intact HSE (ratio depending on skin donor used). This clearly illustrates that TEWL measurements in vitro are not predictive

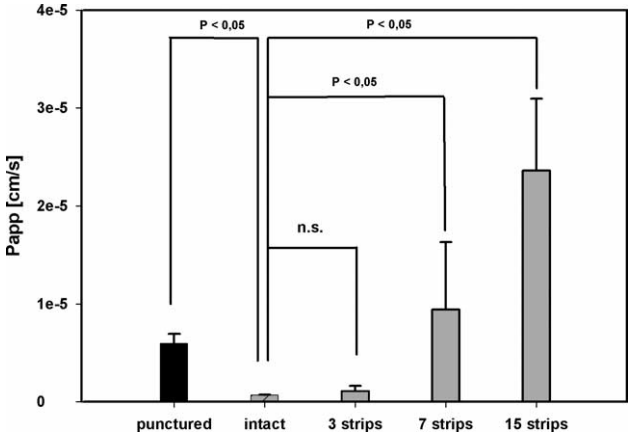

Fig. 6. TEWL values of intact, punctured and stripped HSE (all groups $n=6$).

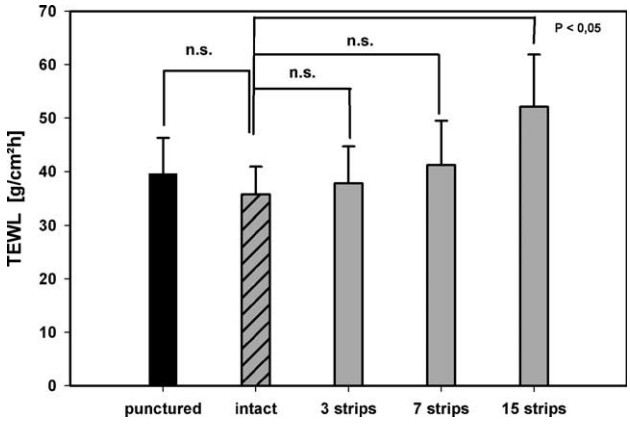

Fig. 7. Papp values of permeation experiments with intact, punctured and stripped HSE (all groups $n=6$).

for drug permeation in vitro even though a local damage of the epidermal barrier function is present.

In the experiments with tape stripped HSE, which focused on reducing the barrier function over the whole surface of the HSE, a statistically significant difference of the TEWL values could only be detected between intact HSE and HSE stripped 15 times (Fig. 6). These results are in accordance with results reported by Pirot [22] who showed that the TEWL rises exponentially when the skin barrier is gradually removed by tape stripping. Furthermore, our findings are in line with results reported by Chilcott who found a significant increase of the TEWL with full thickness pig skin stripped 15 times [23]. In addition, for three and seven times stripping only a slight increase of the TEWL value could be identified. However, these values were not statistically significant different from the intact HSE. Concerning the permeation experiments with gradually weakened barriers produced by tape stripping the application of seven and 15 strips caused a statistically significant increase of the Papp values (Fig. 7). This effect was more pronounced for the 15 times stripping experiments than for the seven times one. The weakening of the barrier of the SC by three strips could not be detected as statistically significant different from intact HSE by transport experiments. However, a trend to higher Papp values with a broader standard deviation was apparent, indicating a minor damage of the barrier.

In summary, TEWL measurements could only detect some relatively severe damage of human HSE sheets in vitro, e.g. induced by tape stripping 15 times. Lighter damage, e.g. done by stripping with three or seven tapes only or by puncturing one hole, could not be determined. However, all damaging procedures, except stripping with three tapes resulted in an increased drug permeation in vitro across human HSE sheets.

5. Conclusion

The OECD guideline 428 [2] requests skin integrity tests for in in vitro diffusion experiments and suggests among other methods the measurement of the TEWL as a possible tool to assess the integrity of the SC barrier. However, the data presented here suggests that this method used in the described experimental design is very limited in its potential to correctly evaluate the barrier integrity in in vitro permeation test systems based on static FDCs. Only a relatively strong damage of the barrier function could be detected, while drug transport was already influenced by slight barrier changes, which a barrier integrity test should be capable of detecting. Therefore, the TEWL measurement does not seem to be an appropriate tool to routinely check skin integrity in human HSE in vitro.

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