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Circadian and Ultradian (12 h) Variations of Skin Blood Flow and Barrier Function in Non-Irritated and Irritated Skin—Effect of Topical Corticosteroids

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The skin is the organ that receives the greatest exposure to light and shows a high-amplitude circadian rhythm in epidermal cell proliferation. We have previously demonstrated that the skin barrier function has a significant circadian rhythm. Corticosteroids (CS) are the most commonly used topical treatment in dermatology. Time-dependent differences in their efficacy and side-effects would be of considerable interest. The aims of the current study were to examine time-dependent cycles in the effect of topical CS application in healthy and irritated skin on skin blood flow and its relationship to barrier function. Twenty clinically healthy, diurnally active subjects were examined at eight and nine time points over a 24 or 28 h span respectively, using non-invasive skin bioengineering techniques of laser Doppler imaging, a transepidermal water loss (TEWL) device and a skin thermometer in a 28 h session. The results of this current study demonstrate circadian and ultradian (12 h) variations in skin blood flow. A significant correlation was found between skin temperature and skin blood flow but not with TEWL. Circadian and ultradian rhythms are maintained during treatment with high-potency and mid-potency CS in healthy skin. These rhythms persist during stratum corneum disruption with and without CS application.

Key words: circadian rhythm/topical corticosteroids/ultradian rhythm
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The human organism shows an intricate time structure consisting of rhythms in multiple frequencies superimposed upon trends like child development and aging (Touitou and Haus, 1992). Circadian rhythms of about 24 h periods that are found ubiquitously in human cells, tissues, organs, and in the organism as a whole are prominent. Many circadian rhythms are genetically fixed and synchronized by light perceived by the retina and the light stimulus related to the suprachiasmatic nuclei (SCN) of the hypothalamus, which acts as a central pacemaker that regulates many rhythmic functions in the body (Rietveld, 1992; Gilette and Tischkan, 1999). The skin is the organ that receives the greatest exposure to light and shows a high-amplitude circadian rhythm in epidermal cell proliferation. This leads to the possibility of an interaction of local oscillator functions in the skin with light as the environmental synchronizer (Zanello *et al*, 2000; Kawara *et al*, 2002). Circadian and other rhythms have been found in a variety of skin-related variables. One of the most important roles of the skin is to generate a semipermeable barrier, to protect against transcutaneous water loss. This barrier is located in the stratum corneum. Recently, we and others (Yosipovitch *et al*, 1998; Denda and Tsuchiya, 2000; Le Fur *et al*, 2001) have shown a time-dependent variation in the stratum corneum barrier of human skin. These studies suggest that the skin permeability barrier is higher during the night and evening than in the morning, and that the resorption, efficacy, and side effects of many drugs applied as patch, gel, or in other forms to the skin surface may vary as a function of the time of treatment. Another factor that may affect the efficacy of topical application is the skin blood flow rate, which has recently been shown to be circadian periodic with a peak during the late afternoon and night hours (Smolander *et al*, 1993; Caspary *et al*, 1997).

Topical corticosteroids (CS) are the most commonly used drugs in dermatologic medicine. Time-dependent differences in their efficacy and side effects would be of considerable interest. A large-amplitude circadian rhythm in the percutaneous absorption of CS may have practical implications for topical CS and other topical agents administration. When the stratum corneum is damaged by tape stripping, a series of reparative processes is immediately accelerated and the barrier recovers to its original level (Elias and Menon, 1991). This process includes exocytosis of lipid containing lamellar bodies in the upper epidermis lipid synthesis and metabolism. Whether this process is time dependent is important in common clinical situations

Abbreviations: CS, corticosteroids; TEWL, transepidermal water loss; LDPI, laser Doppler imager; SCN, suprachiasmatic nuclei; MESOR, midline estimating statistic of the rhythm; PLSD, protected least significant difference

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such as irritant dermatitis and atopic dermatitis where the barrier function is disrupted. Other factors that may influence barrier permeability are short-term application of topical CS, psychological stress, and sex hormones (Garg *et al*, 2001; Kao *et al*, 2001, 2003). Therefore, time-dependent variations in skin irritancy due to application of CS would be of clinical importance.

In this study, we examined the possible connection between timing of skin physiologic events in stratum corneum and skin blood vessels in relation to application of CS to healthy skin and irritated skin. The specific objectives were to determine (1) whether the vasoconstrictive effect of topical CS application is time dependent in healthy skin, (2) whether the skin irritation by tape stripping has a significant time-dependent effect on barrier function, and (3) whether the effects of topical application of CS in irritated skin are time dependent. The potential clinical implications of the results are discussed.

Results

Study 1

Basal skin blood flow demonstrates circadian and ultradian rhythmicity The basal skin blood flow shows a circadian variation in measurements both over the proximal and distal forearm with a rhythm described by 24 h population mean cosinor ($p = 0.04$, $p = 0.02$, respectively). Superimposed upon the circadian periodicity is an ultradian rhythm of about 12 h, which is expressed in the chronograms both from the proximal and from the distal forearm measurements but reaches statistical significance only in measurements of distal forearm ($p = 0.01$). Twenty-four percent of the total variability can be explained by this ultradian rhythm.

The time-dependent variations in the measurements were confirmed by one-way ANOVA for the absolute and normalized data over the distal forearm and the normalized values over the proximal forearm.

The acrophase of the 24 h cosinor curve best fitting to the data was 18:56 (95% CI 12:20–22:36) for the proximal forearm and 18:56 (95% CI 14:56–23:52) for the distal forearm. The peaks of the superimposed 12 h ultradian variations were between 13:00 and 14:00 and between 01:00 and 02:00. Of interest is the deep trough of the circadian rhythm with the lowest blood flow in both measurements found at 08:00 (Fig 1).

The circadian rhythm of skin blood flow is maintained under treatment with CS The basal measurements obtained were used for comparison with the values obtained after CS application at comparably located sites. The circadian mean in the clobetasol-treated skin sites was about 13% lower than in the untreated sites as examined by a paired *t* test (paired *t* value $= 5.8$; $p = 0.001$) (Fig 1). The circadian amplitude remained similar, 0.04 V (95% CI 0.01–0.06) at the treated site *versus* 0.03 V (95% CI 0.003–0.074) at the untreated sites. The circadian acrophase in the population mean cosinor of the clobetasol-treated skin site was 18:28 (95% CI 14:32–21:12), with the highest values measured at 14:00. A 12 h rhythm was not statistically verified at the

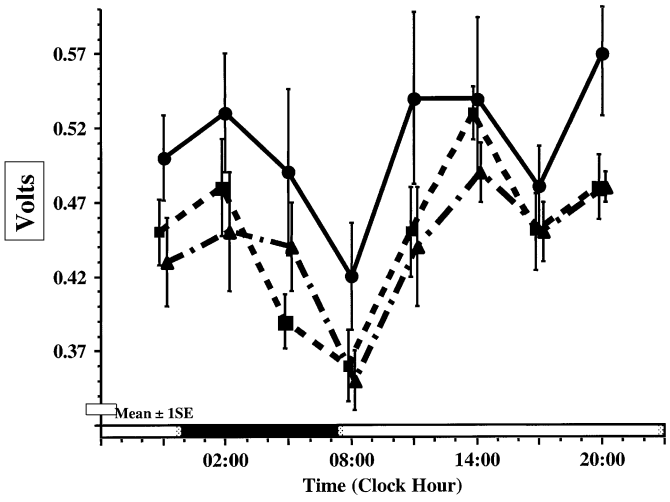

Figure 1
Time dependent variations of skin blood flow on the volar forearm. Measurements were performed in nine study subjects at 3 h intervals during a 24 h period. Data presented of baseline measurements (•), after application of mometasone (■) and colbetasol (▲). The mean values of each group of subjects were determined for each variable at each time point and are shown with their standard errors as time plots. Time dependence was found; a deep circadian trough at 8:00 was noted in baseline as well as in both treatment arms. Peak flows were noted at 20:00 for baseline measurements and smaller ones at 14:00 for all groups. Analysis by the cosinor method detected circadian rhythms of 24 h in all three groups and ultradian rhythms of 12 h in the mometasone-treated skin (see also Table I). The light-off period is indicated as a bold line on the time axis.

$p < 0.05$ level by cosinor (Table I and Fig 1). As in the baseline measurements, there was a deep circadian trough at 08:00 in the clobetasol-treated skin, which in the Fisher PLSD (*F*) test was significantly different from the measurements at all other times. The CS effect (difference baseline–treated) was recognizable in the night and morning measurements (20:00–12:00) but not in the measurements at 14:00–18:00.

At the mometasone-treated site, the circadian mean was similarly lower by about 12% as examined by a paired *t* test (paired *t* value $= 4.9$; $p = 0.001$). The peak blood flow was at 14:00 with the nadir at 08:00 (Fig 1). The circadian rhythm is detectable by the population mean cosinor with an amplitude of similar extent as the baseline values 0.05 V (95% CI 0.03–0.06) in the treated site *versus* 0.03 V (95% CI 0.003–0.07) in the untreated sites. Thirty percent of the total variability is explained by the 24 h rhythm. The circadian acrophase also occurs during the early evening (18:00, 95% CI 16:28–19:56).

In the mometasone-treated sites, there is a 12 hour rhythm component superimposed upon the circadian rhythm, which accounts for 40% of the total variability encountered and shows peaks around 02:00 and 14:00. A deep trough of the circadian variation is found at 08:00. No significant changes were noted in skin blood flow as measured by LDPI between the types of corticosteroids. A steroid effect (difference baseline-treated skin) was recognizable in the night and morning measurements (20:00–12:00) but not in measurements at 14:00–18:00 in both treatment groups. There were no differences between the "mid-potency" (mometasome) and the "high-potency"

(clobetasol) steroid upon skin blood flow under the conditions of this study.

Forearm skin temperature correlates with skin blood flow There was a positive correlation between basal skin blood flow and skin temperature on the forearm ($r = 0.28$, $F = 6.02$, $p = 0.02$), which became more prominent after treatment with colbetasol ($r = 0.33$, $F = 8.8$, $p = 0.004$) and with mometasone ($r = 0.35$, $F = 9.9$, $p = 0.002$).

No relationship was noted between barrier permeability as expressed by TEWL and the maximal effect of corticosteroids.

Study 2

Circadian rhythm of barrier function is maintained following tape stripping and after pre-treatment with mometasone After skin irritation, TEWL showed a similar time-dependent pattern to baseline measurements as in intact skin (Fig 2). As expected after tape stripping irritation, the TEWL is, at all time points measured, markedly elevated over the values obtained in undisturbed skin. Cosinor analysis does not attain a statistically significant description of a 24 h or a 12 h rhythm $p = 0.06$ (Table I). There is however a statistically significant difference between peak and trough values in an *F*-test. Pre-treatment with mometasone slightly reduced the elevated TEWL after skin irritation during the night hours only, with no effect in the measurements between 14:00 and 20:00. There was a circadian variation which, apart from an earlier acrophase, was similar to that seen in untreated skin (Fig 2). The absolute values did not allow statistically significant 12 or 24 h rhythm detection, but the one-way ANOVA for time recognized differences between time points (Table I). The peak times of TEWL in the irritated skin with and without pre-treatment are similar to the time found in study 1 in non-irritated and non-treated skin and those in study 2 baseline measurements in non-treated skin.

Circadian rhythmicity in skin blood flow after tape stripping Skin blood flow measurements in irritated skin had a temporal pattern similar to baseline measurements in intact skin, but a 90% higher mean perfusion (Fig 3). Skin blood flow measurements showed a significant circadian rhythm before and after skin irritation, with the actually measured peaks at 11:00 and the peak time of the best-fitting cosine curve (acrophase) at 12:04 (95% CI 10:48–13:28) and 13:12 (95% CI 08:40–16:56), respectively. This is earlier in the day, a phase advance, in comparison to the basal skin blood flow in study 1. The baseline data did not attain statistical significance in the 12 h cosinor with the absolute values (Table I) but did with the normalized data. A 12 h rhythm in the skin blood flow is not detectable by cosinor analysis after irritation (Table I). This may be due to the greater variability encountered after skin irritation.

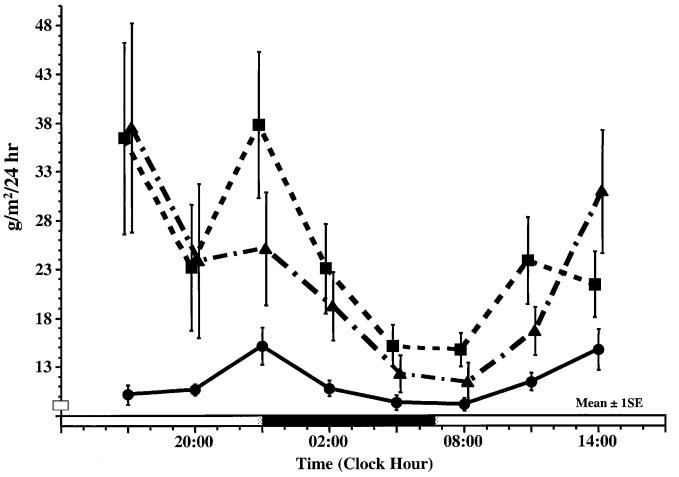

Figure 2
Time-dependent variations of TEWL on the forearm. Measurements were performed on the volar forearm of 11 study subjects at 3 h intervals during a 28 h period. Data presented of baseline measurements (•), after tape strippings (■), and after tape stripping with pre-treatment with mometasone (▲). The mean values of each group of subjects were determined for each variable at each time point and are shown with their standard errors as time plots. A time dependence was found with ANOVA for all three groups, and population mean cosinor for 12 h rhythm was noted in baseline measurements (see also Table I).

Table I. Analysis of variance of time-dependent changes and population mean cosinor of 24 h and 12 h rhythms of TEWL, and skin blood flow with and without topical application of CS on the forearm

Variables	ANOVA p value	Population mean 24 h cosinor p value	Population mean 12 h cosinor p value
TEWL baseline	0.002	ns	0.02
TEWL after tape stripping	0.033	ns	ns
TEWL after tape stripping with pre-treatment with mometasone	0.028	ns	ns
Skin blood flow baseline first study	ns	0.027	ns
Skin blood flow with mometasone	0.0002	0.001	0.004
Skin blood flow with colbetasol propionate	0.05	0.009	ns
Skin blood flow baseline second study	0.0001	0.001	0.05
Skin blood flow after tape stripping	ns	0.015	ns
Skin blood flow after tape stripping in pre-treated skin with mometasone	0.001	0.002	ns

ns, not significant.

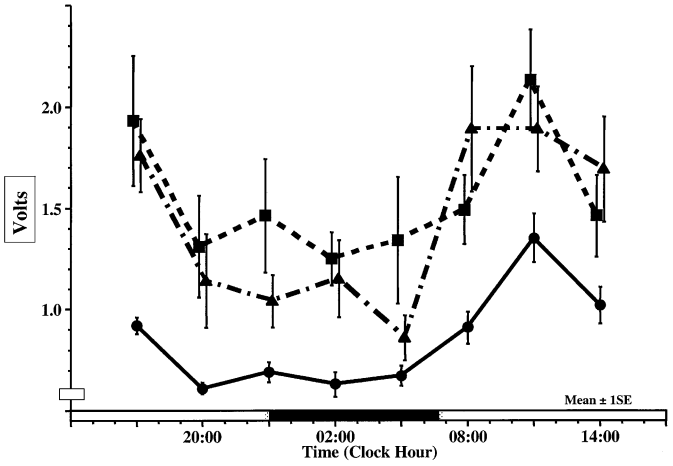

Figure 3
Time-dependent variations of skin blood flow in the volar forearm after tape strippings (■), with pre-treatment with mometasone (▲) *versus* baseline measurements (•). Measurements were performed in 11 study subjects at 3 h intervals during a 28 h period. The mean values of each group of subjects were determined for each variable at each time point and are shown with their standard errors as time plots. Skin blood flow had a similar temporal pattern in tape-stripped skin with and without mometasone to baseline skin blood flow. Peak blood flow was noted at 11:00. The population mean cosinor for 24 h rhythm was noted in all three groups. A 12 h rhythm was not noted after skin irritation (see also Table I).

Skin blood flow after tape stripping with pre-treatment by mometasone maintains circadian rhythmicity Skin blood flow in irritated skin after pre-treatment with mometasone 0.1% had a similar time-dependent pattern as the baseline measurements and untreated irritated skin (Fig 3). There was a highly statistically significant circadian rhythm in the pre-treated subjects without a significant 12 h variation (Table I). The acrophase (12:32 [95% CI 10:16–15:00]) was similar to that of the untreated irritated skin and the baseline measurements. A lowering of LDPI by mometasone was observed during the night-time in the pooled data between 20:00 and 05:00 (paired t-value $= 3.06$, $p = 0.003$). There was no such effect during daytime.

Discussion

The results of this study show circadian and ultradian variations in skin blood flow and barrier function, which are maintained during CS treatment in healthy and irritated skin.

The time-dependent variations in skin blood flow are in agreement with recent studies on healthy skin and the skin of patients with mild vascular disease (Houben *et al*, 1994; Caspary *et al*, 1997). The mometasone furoate- and clobetasol propionate-treated sites showed similar circadian and, in addition, 12 h ultradian variations. Interestingly, although clobetasol propionate is considered to be a more potent CS than mometasone furoate, the effects of both drugs on skin blood flow were similar. LDPI blood flow measurements have been shown recently to provide reliable and accurate data to determine the degree of vasoconstriction (Sommer, 1998), and it seems this technique provides an accurate assessment of the vasoconstrictive effects of CS.

The most consistent observation is the daily low cutaneous blood flow during the morning hours. The highest values are found usually during the afternoon and early evening, with a second peak in the late evening before sleep onset. There is a wide variation in the acrophase of the cosinor as expressed by the large 95% confidence interval. This is in part due to the superimposed ultradian (12 h) variation, with a second peak prior to sleep onset that leads to a non-sinusoidal shape of the circadian variation. The secondary peak, which by cosinor analysis appears to indicate a 12 h rhythm, may be an expression of the shift in heat distribution from the core to the periphery (as measured by an abrupt increase in peripheral skin temperature taking place before sleep onset) (Krauchi and Wirz-Justice, 2001). A secondary peak is not recognized by the same statistical procedure in temperature obtained at the forehead of the same patients (data not shown). The low skin blood flow in the morning may be related to high plasma catecholamine levels and the related increased peripheral resistance found during the early morning hours (Elherik *et al*, 2002).

These results differ from the study by Pershing *et al* (1994), who described a circadian difference in the action of topical betamethasone dipropionate application at 16:00 that produced more extensive changes in skin blanching as a measure of vasoconstriction than morning application at 09:00 in the same subject. The methods used in the latter study however were based on visual measurement with a chromameter to evaluate the vasoconstrictive effect of CS and did not measure blood flow. Furthermore, the limitation of the study to only two time points does not allow the description of a circadian rhythm.

The results of this study reveal time-dependent differences in skin physiology, which may affect the absorption of topical drugs in undisturbed as well as irritated skin. Both therapeutic and toxicologic considerations have to be considered. The maximal therapeutic effect, as suggested by the steroid effect upon blood flow in irritated skin, seems to occur after application during the later evening hours. This corresponds in timing to the nocturnal rise in inflammatory activity with early morning peak in IL-6 as inflammatory mediator (Haus and Smolensky, 1999), which has led to the consideration of a dosing of CS around 02:00 in patients with rheumatoid arthritis (Arvidson *et al*, 1997). From this viewpoint, it may be advantageous to dose topical CS at night in acute inflammatory states where a high concentration of the drug is required and a rapid drug effect is desired in the late afternoon or evening, rather than giving the CS in the morning when a lesser immediate effect would have to be expected. Similar dosing times have been found to be advantageous in the anti-inflammatory steroid medication of bronchial asthma (Kraft, 1999; Smolensky *et al*, 1999). Other clinical implications of this study are that the patients' biologic timing and the time patients are examined for skin blood flow should be taken into account in studies as well as clinical investigations. In addition, we found that the peripheral skin perfusion seems to follow the same overall pattern as body temperature and is involved most probably in the circadian rhythm of body core temperature (Houben *et al*, 1994). This has a significant effect on transdermal drug delivery. It has been previously shown that heat-induced local vasodilatation and acceleration of skin blood flow affect drug passage

through the skin and diffusion from cutaneous and subcutaneous tissues to systemic circulation (Shomaker *et al*, 2001). It has to be realized, however, that the circadian rhythm of the pharmacodynamics of a drug does not necessarily follow the circadian-periodic changes in its pharmacokinetics (Lemmer and Bruguerolle, 1994).

Our results are of relevance to skin pharmacology. The recognition of a significant circadian variation in skin blood flow and skin permeation, which is maintained under barrier disruption and under topical CS treatments, might result in the development of treatment strategies for topical applications taking into account chronopharmacology.

Materials and Methods

Subjects for study no. 1: Nine clinically healthy Chinese (four men and five women, 31 $\pm$ 4.5 y of age) (mean $\pm$ SD, range 24–36), who gave informed consent, participated in a 24 h session.

Subjects for study no. 2: Eleven clinically healthy volunteers (eight Chinese and three Caucasians), who did not participate in study number 1 (seven men and four women, 30.5 $\pm$ 4.7 y of age) (mean $\pm$ SD, range 25–39), who gave informed consent, participated in a 28 h session. Both studies were approved by the National Skin Center ethics committee. The study was performed in accordance with guidelines of the Helsinki Principles.

The subjects, all non-smokers, had no skin disease or atopic history, nor had they used topical or systemic CS preparations in the previous 1 mo. None of the women were pregnant or taking oral contraceptives for at least 6 mo. All women were chosen to be in the luteal phase of their menstrual cycle during the study (Reinberg *et al*, 1996), and were instructed to refrain from applying soaps or moisturizers on the area studied at least 24 h before the study. All followed a diurnally active living pattern, with rest during the night on an average from 23:00 to 07:00. During the study, lights were turned off at 22:30. Study no. 1 occurred during January 2000, and study no. 2 during the same month a year later.

During both studies, the climate and environment were well controlled in environmental temperature (22°C $\pm$ 1°C) and humidity (50%–60%). All subjects shared the same meals (spicy food, alcohol, and hot beverages were not permitted during the study) and engaged in comparable mental and physical activities and rest periods during the session with a 30 min adaptation period before each sampling. The patients were seated in a temperature-controlled room and were instructed not to eat or drink during that period. Non-strenuous activities such as reading, watching television, and writing were allowed. The patients wore the same clothes day and night. They were instructed to wear short-sleeved shirts, leaving the volar part of the forearm free. They slept with light blankets, and were instructed not to apply water on the investigated area during the study. All measurements were performed while the examinees were in a recumbent position and their forearms in a horizontal position.

Protocol study no. 1 We measured the effect of a long-acting mid-potency CS, mometasone furoate 0.1% (Elomet ointment, Schering Plough, Singapore, Singapore), and a high-potency corticosteroid, clobetasol propionate (Dermovate ointment, UCB), UCB, Smyrna, GA on skin blood flow using a scanning laser Doppler imager (LDPI, LISCA, Linkoping, Sweden), which rapidly measures blood flux over a 4 cm^2 area without contact with the skin surface. All measurements were conducted at a scanning distance of 20 cm. The mean perfusion was calculated from 264 pixels. The borders of the measured areas were marked before the measurement. Baseline measurements prior to drug application were made both over the proximal and over the distal forearm to ensure that there are no significant differences in site that can influence the analysis of the results in sites of drug application.

Skin temperature was measured with an infrared thermometer type K model (AI 38) (Northlab, Singapore) with a sensitivity of 0.1°C. Transepidermal water loss (TEWL) was measured with a TEWL device (Dermalab, Broomall, Pennsylvania). TEWL values were registered in g per m^2 per h after equilibration of the probe on the skin for 60 s. Measurements were conducted on the volar part of both forearms. Eight circular sites 2 cm in diameter were outlined and numbered 1–8 with a skin marking pen. There was a 2 cm distance between the sites. The drugs were applied 3 h before each measurement. Mometasone furoate 0.1% ointment was applied to one of the marked circles and clobetasol propionate 0.1% ointment to the contralateral forearm. Every 3 h, 20 μL of each cream was pipetted on the center of a different circle chosen randomly within the test area and gently massaged for 30 s. Measurements of skin blood flow laser Doppler imaging were performed with the PIM Lisca on both sites of drug application and also in two untreated sites—one on the proximal forearm and one on the distal forearm—to ensure that there are no significant differences in measurements between the sites of application. Measurements were conducted 3 h after drug application at the following time points: 23:00, 02:00, 05:00, 08:00, 11:00, 14:00, 17:00, and 20:00. Measurements of TEWL and skin temperature were conducted on untreated sites at the times mentioned above.

Protocol study no. 2 On both volar forearms on marked sites, barrier disruption was achieved with 30 tape strippings with cellophane scotch tape (3M, St Paul, Minnesota) every 3 h. For each subsequent measurement of barrier function, a different site was tape stripped and TEWL was measured (altogether eight sites). On the left forearm, pre-treatment with mometasone furoate 0.1% ointment was applied 3 h before tape stripping each site. Skin blood flow and TEWL were measured during the 24 h span every 3 h consecutively at the following time points: 17:00, 20:00, 23:00, 02:00, 05:00, 08:00, 11:00, and 14:00, 17:00 on treated and untreated sites of tape-stripped skin and on a non-stripped skin site.

Statistical analysis All values were analyzed in absolutes and normalized in percent of the 24 h mean value. The percent of mean emphasizes the periodicity of the variables and eliminates differences in levels between subjects. The mean values of each group of subjects were determined for each variable at each time point, and are shown with their standard errors as time plots in Figs 1–3. Single cosinor curve fits (Nelson *et al*, 1979) were performed on each subject's data over a spectral window, with periods from 6 to 28 h (in 0.5 h intervals), to determine the best-fitting cosine curves suggesting ultradian (less than a 20 h period) and/or circadian (20.5–28 h periods) variations. Since the best-fitting curves in the subjects clustered around 12 and 24 h, the single cosinor results from the 12 and 24 h fit for each subject were used as input for a population mean cosinor for the group (Nelson *et al*, 1979). Population mean cosinors were performed on men and women separately and on the entire group. Because of the small numbers, the data presented are on the entire group for each study (study 1: nine subjects; study 2: 11 subjects). The population mean cosinor provides the rhythm parameters of the rhythm-adjusted mean (**m**idline **e**stimating **s**tatistic **o**f the **r**hythm = MESOR), the amplitude (the excursion from MESOR to the peak or trough of the rhythm representing half the peak–trough interval of the fitted cosine curve), and the acrophase (the peak time of the best-fitting cosine curve) in relation to local midnight as a phase reference. Rhythm description by cosinor was accepted when the null hypothesis for amplitude was rejected in an *F* test at/below the 0.05 level. In addition, the data of the group were subjected to analysis of variance (ANOVA) in one-way ANOVA for time. p values below 0.05 were accepted as indicating statistical significance. The difference between single time points was determined by the *F* test (Fisher PLSD (protected least significant difference) where appropriate.

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