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Ex vivo Skin Permeation of Betulin from Water-in-Oil Foams

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Key Words
Betulin · Water-in-oil foam · Permeation · Injured skin · Porcine skin

Abstract
Triterpenes of the outer bark of birch are known to improve wound healing. An oleogel with these triterpenes as active principle is approved by the European Medicines Agency. As foams can be applied without touching the skin, they might be an advantageous application form. A comparable wound-healing effect can be expected when the permeation flux of the triterpenes from different types of formulations, namely oleogels, water-in-oil emulsions and water-in-oil foams, is similar. The tested formulations were based on three lipids (medium-chain trigylcerides, sunflower oil and paraffin) which differ in their polarity and solvent power for the triterpenes. Infinite dose permeation experiments were performed using porcine skin which was injured by either tape stripping or skin grafting. The results showed that steady-state permeation flux and lag time depend clearly on the depth of the skin lesion. Moreover, it was substantially affected by the lipid used as basis for the different formulations. In contrast, the different formulation types showed a comparable permeation behaviour leading to the conclusion that all formulation types can be used alike for the treatment of wounds, and the results that have already been obtained with oleogels can be directly translated to the foam with its superior use properties.

Introduction

Many botanical extracts are known to have positive effects in skin treatment [1–3]. Triterpenes are non-polar biologically active secondary plant metabolites. Their various pharmacological properties like anti-inflammatory, antiviral and wound-healing effects are well investigated and specified in the literature [4–8]. Also in cancer treatment triterpenes are known as potent agents [9, 10]. These substances are widely distributed in plants but only the outer bark of the white-barked birch contains up to 34% (w/w) betulin, a pentacyclic, lupan type triterpene with two polar hydroxyl groups located on opposite sides of the molecule [11]. A well-characterized triterpene dry extract from the outer bark of birch ('triterpene extract', TE) is obtained by accelerated solvent extraction with n-heptane and contains about 80% (w/w) betulin [12]. Further identified main constituents of this dry extract are lupeol, erythrodiol, betulinic acid and oleanolic acid [13] (table 1).

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Table 1. Chemical composition and physical characteristics of the TE used

Chemical composition	Specific surface area	Particle size D50%
81.6% betulin 3.84% betulinic acid 2.08% lupeol 1.05% erythrodiol 0.97% oleanolic acid 0.52% metulinic acid methylester 9.94% unidentified substances	42 ± 0.4 m^2/g	5.8 μm

Triterpenes from the outer bark of birch have been shown to improve wound healing. Wounds and injured skin of several types have been examined: actinic keratoses, surgical skin lesions and necrotizing herpes zoster [14–17], and the benefit of this dry TE in wound healing has been underlined. Besides, the molecular mechanism and ex vivo wound-healing models have been investigated [18].

A beneficial further feature of the TE is its galenical properties: Mixed with oils, the birch bark extract forms stable oleogels, which can absorb up to 60% of water forming a water-in-oil (w/o) emulsion [13, 19, 20]. At the time being, available topical application forms are oleogels [13] and cosmetic w/o creams (Imlan Creme pur, Birken AG). The oleogel containing sunflower oil got the European marketing authorization in January 2016 [21].

However, applying such dermal formulations to injured skin will cause significant physical stress and can be painful to the patient. A more advantageous form might be foams, which can be applied almost touchless. Foams are formulations preferred by patients in the treatment of skin diseases [22–24] hence leading to a better patient compliance, which is important especially for the treatment of chronic wounds. Usually foams are based on oil-in-water emulsions where the propellant (commonly propane/butane mixtures) is mixed with the dispersed lipid phase of the emulsion. In contrast, the foams which have been investigated in this study are based on w/o emulsions which are the preferred emulsion type using TE. To this end, TE stabilized w/o emulsions were charged with carbon dioxide as propellant.

This concept allows to combine the advantages of a touchless application with those of the healing effects of the birch bark triterpenes in a formulation which actually contains only TE, oil and water. Medium-chain triglycerides (MCT), sunflower oil and paraffin were chosen because they affect wound healing differently and because of their different polarities which in turn directly influence their interaction with TE. MCT shows good wound-healing properties even without TE. Oleogels with sunflower oil improve wound healing significantly [25]. Paraffin was used as non-polar reference. Permeation experiments were conducted to evaluate whether the availability of betulin is the same from all formulations and the advantageous use properties of the foam can be combined with the healing properties of the yet existing oleogel. For the permeation experiments, artificially injured skin was needed. A very common method to prepare skin for studies of this kind is tape stripping [26, 27] where the cell layers of the stratum corneum are successively removed strip by strip. The other preparation method is inspired by the wounds of skin graft donor sites [15], where the outer layers are removed with a dermatome.

The aim of this study was to predict the wound-healing effect of the w/o foams containing TE of the outer bark of birch by comparing the betulin permeation from these novel formulations to the permeation of betulin from oleogels which have already been characterized with respect to their ability which have proven to promote wound healing [16]. To this end the skin permeation rates of betulin, the main constituent of the TE, from oleogels, w/o creams and w/o foams were compared. Special emphasis was put on the influence of (1) the depth of the skin lesion of artificially injured skin and (2) the different types of oils used as carrier for the TE.

Materials and Methods

Materials

TE from the outer bark of birch was obtained from Birken AG, Niefern-Öschelbronn, Germany (table 1). Sunflower oil (Caesar & Loretz GmbH, Hilden, Germany), MCT (Myritol 318, BASF, Ludwigshafen, Germany), light liquid paraffin (Hansen & Rosenthal KG, Hamburg, Germany), CO_2 (Westfalen, Münster, Germany), hydroxypropyl-β-cyclodextrin (Kleptose HP oral grade, donated by Roquette, Lestrem, France), Parafilm® (Bemis Company Inc., Oshkosh, Wis., USA), sodium chloride, disodium hydrogen phosphate and potassium dihydrogen phosphate dihydrate were of European Pharmacopoeiae grade, acetonitrile of HPLC gradient grade.

Preparation of the Foams

For all of the tested formulations, paraffin, sunflower oil or MCT served as the basis of the different oleogels. These oleogels containing 10% (w/w) TE were prepared by dispersing the TE in the respective oil using an Ultra-Turrax T25 (IKA, Staufen, Germany) at 8,000 r.p.m. for 3 min. After a storage period of 24 h, the same amount of water was added to the oleogels using a syringe-

to-syringe technique yielding homogeneous w/o emulsions. 50 ml of the emulsions were filled in aluminium aerosol cans and charged daily with CO_2 in order to obtain a constant equilibrium pressure of 5 bar after 5 days.

Viscosity Measurements

The apparent shear viscosity of the oleogels was determined at 100 s^{-1} and 25°C using a Physica MCR 501 rheometer (Anton Paar GmbH, Graz, Austria) equipped with a 25-mm cone/plate geometry and a gap of 0.048 mm. Measurements were taken 24 h after the preparation of the oleogels and were performed in triplicate.

Preparation of Porcine Skin

The pig ears were washed with isotonic saline solution, cleaned of blood with cotton swabs and dried. The excised postauricular skin was wrapped in aluminium foil and stored at –30°C. On the day of the experiment, it was thawed at room temperature and was pinned to a styrofoam block. If not otherwise pretreated as described below, the porcine skin was then cut with the dermatome (Dermatom GA 630, Aesculap AG & Co. KG) to a thickness of 0.8 mm.

Injuring Skin

Superficial wounds are limited to the outer skin layers and are often caused by abrasion. Depending on the depth of the abrasion process, the different layers of the epidermis or the dermis can be involved. The severity of the injury has a clear impact on the healing process and also on the penetration of an active agent as the barrier properties of the remaining skin tissue vary. In order to simulate two kinds of abrasion porcine skin was 'injured' for this study in two different ways: firstly, by skin stripping to remove the horny layer of the skin and, secondly, by skin grafting using a dermatome to remove the outer 200 μm of the skin.

To this end, the skin was prepared as previously described and subsequently injured by either of the following two methods.

For tape stripping, the skin on the styrofoam block was pressed to the tape (Tesa No. 4124, Beiersdorf AG, Hamburg, Germany) and stripped in one quick move. This procedure was repeated 20 times.

For skin grafting, 0.2 mm of the outermost layers of the skin were removed by the means of a dermatome (Dermatom GA 630, Aesculap AG & Co. KG), cutting directly in the living layers of the skin.

All three kinds of skin (untreated, skin after tape stripping and grafted skin) were finally dermatomed to a constant thickness of 0.8 mm. The uniform severity of both types of injuries was confirmed by light-microscopic examination.

Microscopic Examination of Injured Skin

To determine the severity of the injury, microscopic images were taken. The skin was treated as described before and frozen in liquid nitrogen. Cross-sections were cut with a cryomicrotome (HM 560 Cryo-Star; Thermo Fisher Scientific Inc., Langenselbold, Germany). The sections had a thickness of 50 μm and were stained with haematoxylin and eosin before taking microscopic images (Microscope Axio Imager Z1, Carl Zeiss, Jena, Germany).

Ex vivo Permeation Experiments

Permeation experiments were performed using modified Franz-type diffusion cells (Gauer Glas, Püttlingen, Germany) [28] with a receptor volume of 12 ml. Phosphate-buffered saline, pH 7.4, was used as receptor fluid with 10% hydroxypropyl-β-cyclodextrin to enhance the solubility of betulin. The receptor fluid was preheated to 32°C and filled into the diffusion cells. Skin samples (thickness 0.8 mm, diameter 2.5 cm) were obtained from porcine skin that was either untreated retaining the natural skin barrier or 'injured' by either tape stripping or by skin grafting. The donor compartment was fitted to the cells, and they were heated to 32°C in a water bath followed by an equilibrium time of 30 min. For infinite dose experiments, 1 g of the formulations was applied uniformly to the porcine skin. The diffusion cells were capped with Parafilm® to avoid water evaporation. The stirring speed of the receptor fluid was 500 r.p.m. Samples of 0.5 ml were taken after 2, 5, 8, 21, 24 and 27 h and replaced by fresh preheated receptor fluid. To obtain the amount of permeated betulin, the samples were analysed via HPLC. The cumulative permeated amount of betulin per area was plotted against the time. The flux of the permeation was calculated using the results from 8 to 27 h. The first two samples were excluded for linear regression as the flux was not yet in a steady state in all the experiments. All experiments were performed in quintuplicate.

Betulin Assay

Betulin was quantified by HPLC using the following system: LC-20A prominence HPLC system (Shimadzu, Duisburg, Germany), HPLC column Nucleosil 100-5 C_{18} EC 125/4, HPLC precolumn Universal RP EC 4/3 (both Macherey-Nagel, Düren, Germany). The temperature for the column was set to 40°C and the flow rate to 1.5 ml/min. The composition of the mobile phase was 70% of acetonitrile and 30% of water. The limit of detection was 0.0491 μg/ml and that of quantification 0.1473 μg/ml. A volume of 20 μl of every sample was injected, and the UV absorbance was measured at 210 nm. The retention time for betulin was approximately 10.3 min.

Statistical Analysis

All data was obtained by repeating the measurements ($n \geq 4$) and analysed by one-way (single factor) analysis of variance followed by the Student-Newman-Keuls test.

Results and Discussion

Microscopic Examination of Differently Injured Skin

The microscopic images in figure 1 show the severity of the damage to the skin after the two different treatments, tape stripping and skin grafting, compared to the untreated, full-thickness skin. The untreated skin shows the typical layer structure of skin including stratum corneum, epidermis and dermis. After tape stripping, the stratum corneum as the outermost layer of the skin has been removed completely from the skin. In contrast, skin grafting leads to a severer damage, cutting directly deep into the living epidermal layers of the skin.

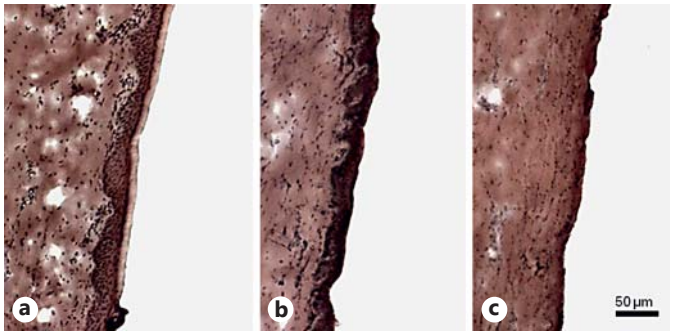

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Fig. 1. Microscopic images of untreated porcine skin (**a**), skin after tape stripping (**b**) and grafted skin (**c**).

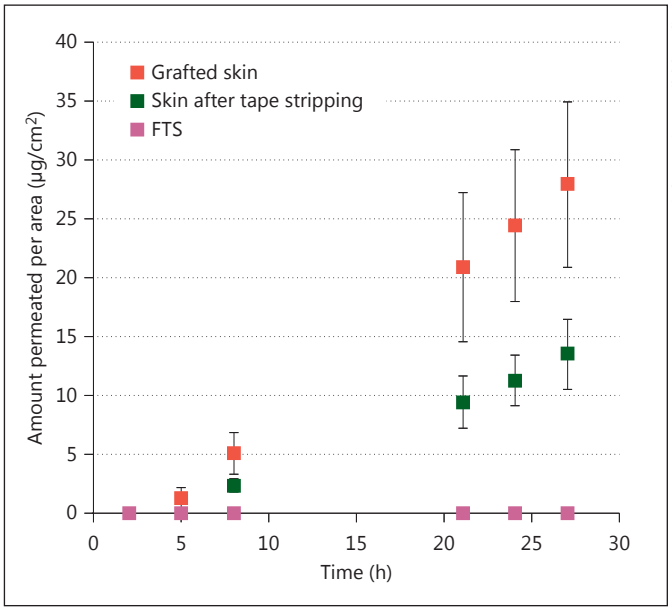

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Fig. 2. Comparison of betulin permeation from sunflower oil oleogels through differently injured skin and full-thickness skin (FTS); n = 5; error bars: standard deviation.

Permeation through Differently Injured Skin

First, permeation of betulin from sunflower oleogels through the different types of skin was investigated. Intact skin displays an almost perfect barrier function and prevents the delivery of xenobiotic molecules like betulin across the skin. Experiments with full-thickness skin revealed a marginal permeation of betulin with a value below the limit of quantification (0.88 µg/cm^2; fig. 2). As expected, permeation flux was significantly higher when the barrier function of the skin was artificially impaired. As tape stripping only removes the stratum corneum, the flux after tape stripping (0.44 ± 0.11 µg/cm^2 × h) was only half the value than through grafted skin (1.13 ± 0.15 µg/cm^2 × h). Furthermore, the lag time, defining the time taken until betulin initially enters the receptor fluid, is shorter for the permeation through the skin damaged by a dermatome compared to stripped skin (4.02 ± 1.02 vs. 6.51 ± 1.48 h). As the flux is inversely proportional to the thickness of the skin, this variable was kept constant throughout all experiments. Therefore it is only influenced by the diffusion coefficient which is dependent on the structure of tissue that has to be crossed. Although after the injury of the skin by tape stripping and grafting the stratum corneum as the major barrier has been removed, there is still a different resistance to betulin permeation. A possible explanation might be that after tape stripping a smooth surface remains whereas grafting cuts directly into the intact tissue and opens additional pathways for the permeating triterpenes. In the context of wound healing this means that the deeper the injury, the more betulin penetrates the skin per unit time, and a more pronounced wound healing effect can be expected with increasing severity of a wound.

Table 2. Interfacial tension, solubility and log P (octanol/water partition coefficient) values of TE with different oils [25]

	Interfacial tension, mN/m	TE solubility, mg/ml	log P
Paraffin	42.7	0.41	3.21
Sunflower oil	25.4	4.4	4.24
MCT	27.1	7.9	4.50

Comparison of Permeation from Oleogels Comprising Different Oils

Solubility and gel strength of TE oleogels depend strongly on the polarity of the used lipid but show no simple correlation due to a complex overlapping of several effects [9, 11]. In order to evaluate whether the nature of the used oil has an impact on betulin permeation, we chose for this study sunflower oil which is of medium polarity and has already been proven to enhance wound healing [15, 16]. As second triglyceride with similar polarity but higher solubility, MCT was selected. Paraffin was selected as an example of a non-polar lipid. The properties of the selected oils are summarized in table 2.

Interestingly, the permeation flux of the oleogels prepared with different oils showed a clear trend regarding the betulin flux which was independent from the severity

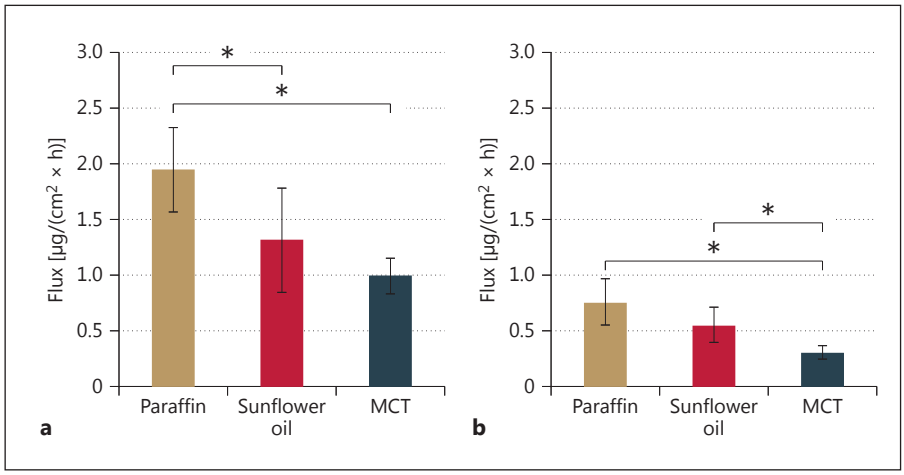

Fig. 3. Permeation flux of three different oleogels through grafted skin (**a**) and skin after tape stripping (**b**); n = 5; error bars: standard deviation; * $p < 0.05$.

Table 3. Viscosities of the different oleogels

Oil phase	Viscosity, η
Paraffin	0.11 ± 0.025
Sunflower oil	0.41 ± 0.028
MCT	0.48 ± 0.026

n = 3; mean ± standard deviation.

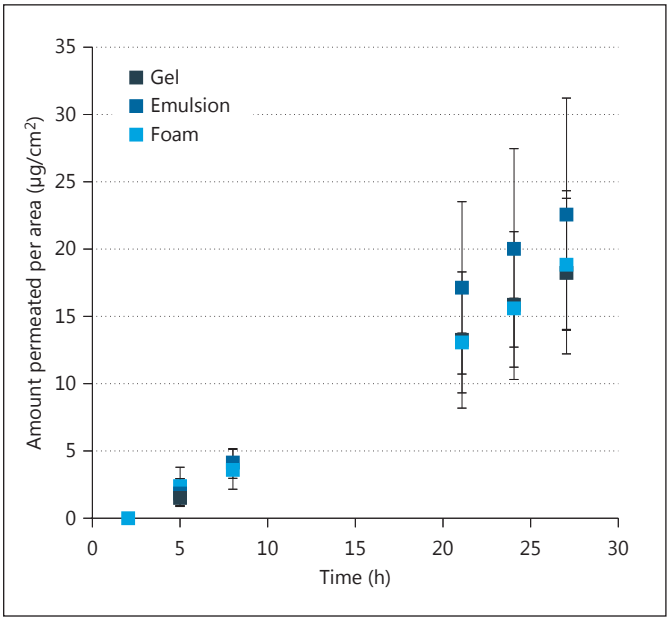

Fig. 4. Comparison of betulin permeation from foam, emulsion and oleogel containing MCT through grafted skin; n = 5; error bars: standard deviation.

of the injury. For grafted skin as well as for skin after tape stripping, the flux increases in dependence on the used lipid phase in the following order: MCT < sunflower oil < paraffin (fig. 3). All oleogels share the fact that the oil is saturated with an excessive amount of TE suspended as solid particles in the oil phase. Consequently, the oil is saturated with betulin, and its activity coefficient in all oleogels is 1. In all cases the concentration in the skin should yield its saturation concentration when distribution equilibrium is achieved. However, skin permeation is a dynamic process and might also depend on the release kinetic of the active agent from the vehicle. Obviously, the different viscosities of the oleogels affect the transport of the active drug to the skin. The respective apparent viscosities are summarized in table 3. Comparing flux and viscosity shows that there is a clear relationship but not a full correlation as rheological measurements characterize the macroviscosity of a system whereas diffusion and release are influenced by the microviscosity [29]. Interestingly, the observed order in the permeation flux with the different oils differs from the release rate measured from equivalent oleogels [16]. This indicates that although the stratum corneum as the predominant skin barrier has been removed for these experiments there is a specific interaction between the oil which is used as vehicle and the injured skin.

Comparison of Permeation from Different Formulations Containing the Same Oil

Finally, the different formulation types (oleogel, emulsion and foam) based on the same oil were examined. As

Table 4. Comparison of flux and lag time of betulin from the different formulations

Used oil	Kind of injury	Formulation	Flux, $\mu g/cm^2 \times h$	Lag time, h
Sunflower oil	tape stripping	gel	0.44±0.11	6.51±1.48
		emulsion	0.36±0.11	5.70±2.18
		foam	0.43±0.14	4.34±2.24
	grafting	gel	1.13±0.15	4.02±1.02
		emulsion	1.15±0.13	3.37±0.64
		foam	1.29±0.25	3.32±1.15
Paraffin	tape stripping	gel	1.02±0.17	4.01±2.07
		emulsion	0.87±0.42	3.21±3.11
		foam	0.98±0.31	2.46±2.29
	grafting	gel	1.95±0.39	4.06±1.29
		emulsion	1.67±0.45	4.82±1.28
		foam	1.45±0.50	5.02±1.88
MCT	tape stripping	gel	0.58±0.17	7.47±0.79
		emulsion	0.76±0.22	6.38±1.97
		foam	0.65±0.22	6.49±1.07
	grafting	gel	0.76±0.34	2.72±2.03
		emulsion	0.98±0.47	3.51±2.05
		foam	0.78±0.20	3.69±1.72

n = 4–5; mean ± standard deviation.

can be seen in figure 4 exemplary for formulations containing MCT and in table 4, all types of formulations revealed almost identical permeation kinetics for betulin when applied to skin with the same pretreatment. This can be attributed to the fact that the oleogel with an excess amount of TE forms the outer phase of all types of formulation and therefore is likewise in direct contact to the skin. As a result, neither water nor CO_2 which when present are located in the inner phase of these formulations do significantly affect permeation flux or lag time.

(Note: The permeation values of oleogel, emulsion and foam are to be compared for the same oil and the same injury only.)

Conclusion

This study showed that foams prepared from w/o creams with TE as an active excipient lead to permeation rates through injured skin which are comparable to those of the corresponding oleogels, which have been proven to promote wound healing. Thus, the foams can be considered an advantageous application form in wound treatment, which combines the positive effects of the birch bark dry extract with the advantages of the application form that allows almost touchless application.

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Statement of Ethics

Pig ears were received from the Department of Experimental Medicine of the University Hospital Tübingen. The live animals were kept at the Department of Experimental Medicine and were sacrificed in the course of the experiments, which were approved by the Ethics Committee of the University Hospital Tübingen. The ears were received directly after the death of the animals. The Department of Pharmaceutical Technology is registered for the use of animal products at the District Office of Tübingen (registration number: DE 08 416 105 221).

Disclosure Statement

The authors report no conflicts of interest.

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