W. Pittermann*, W. Lehmacher**, M. Kietzmann***, H. Mehlhorn****

Treatment Against Blood-Sucking Insects Without Skin Irritation

Keywords: anti-louse shampoo, neem oil, DBE-2, skin compatibility, in-vitro, BUS model, MTT, PGE_2

Introduction

In recent times the increasing incidence of head lice infestations (*Pediculus humanus capitis*, **Fig. 1**) has resulted in a significant impact on social relationships in children's playgrounds and schoolyards and has affected family activities. As the number of lice infected children has increased, some of the anti-louse products on the German and European markets show reduced activity, and other insecticide-containing products are not effective at all, or provoke painful skin sensations. Thus, the surviving lice give rise to new generations of these unpleasant blood-suckers, that are easily transmitted to heads of other family members or playmates, if, while playing, there is head/hair to head/hair contact.

Fig. 1 Light micrograph of a female head louse with an egg – Note the claws at the 6 legs

A major problem in increasing the awareness of louse infestations is the mistaken notion that the occurrence of lice on a child's head is the result of unsanitary conditions. This conclusion leads to social isolation of such children and their families, thus producing distinct barriers to interactions in playgrounds and schoolyards. In fact, numerous studies have confirmed that people from all socioeconomic levels are affected. Thus, there is an obvious need to develop anti-louse products with high efficacy rates, with a pleasant aroma, and minimal or no side effects, since louse bites may provoke

Summary

In recent times the increasing incidence of head lice infestations (*Pediculus humanus capitis*) has resulted in a significant impact on social relationships in children's playgrounds and schoolyards and has affected family activities. Many anti-louse shampoos show reduced activity or irritate the skin. They were ineffective or used in low concentrations due to the painful sensations. A newly developed anti-louse shampoo (Wash-Away®) with neem seed extract (*Melia azadirachta*,10%) as active ingredient was proved in volunteer studies to be effective and skin compatible. In this *in-vitro* BUS-study (modified rinse-off conditions, undiluted open application, MTT-assay, Prostaglandin E_2 (PGE_2) - tissue concentration) the skin compatibility of Wash-Away® was compared with a well known brand (Nivea Silky Shine®), Wash-Away® formulation without active ingredient, the pure neem seed extract and the solvent DBE-2.

The skin compatibility of both market products, Nivea Silky Shine®, Wash-Away® were excellent and quite comparable. No statistically significant differences (MTT/ PGE_2) between the two shampoos at any exposure period (0,5h, 1,0h, 5,0h)could be obtained. The active ingredient neem seed extract does not substantially alter the biological properties of the shampoo formulation regarding skin compatibility. The application of pure neem seed extract and the solvent DBE-2 provokes a low skin compatibility. Additionally the application of DBE-2 increased significantly the tissue concentration of PGE_2.

The experimental set up and the study design using the BUS-model allow comparisons of the skin compatibility of active ingredients, solvents, complete formulations and benchmark products under identical experimental conditions.

small lesions and local inflammation due to severe itching. Newly developed anti-louse products must be formulated for use at least two times within 8-10 days because none of the available products affect the developing larvae in the egg (nits) **(Fig. 2)**. These nits are glued to hair shafts in close proximity to the scalp. As early as 5-8 days after deposition of the eggs by the female, the larvae hatch from the nit and begin blood sucking activity, reaching maturity within another 10 days (1-5). Wash-Away-Laus® is a newly developed anti-louse compound with very good efficacy on head lice.

Fig. 2 Scanning electron micrograph of an egg (nits) of the head louse. These eggs (nits) are glued to hair shafts in close proximity to the scalp

According to the relevant EU Council Directive 76/768/EEC (1976) and the 7th amendment, the producers of cosmetics intended to contact the skin are strictly obliged to rule out possible damage of the skin. This also includes safety considerations of completely formulated products with active ingredients. Besides the active ingredients, other substances may be formulated which are able to penetrate the outermost layer of the skin and may alter the barrier and reservoir function of the horny layer.

For many years, experimental dermatological *in-vivo* studies for cosmetics on animals have been prohibited due to ethical and legal restrictions. Results of volunteer studies such as epicutaneous tests or general use tests do not match all possible and scientifically related questions connected with formulated products. In recent years, a great effort was undertaken to develop animal-free skin research methods (*in-vitro* or *ex-vivo*) to study penetration and cellular reactions such as irritation after skin contact. Without legal and ethical restrictions, biological properties of active and other ingredients or chemicals, as well as various formulated products, can be studied, tested, and their results may be directly compared (6).

The standardized BUS-skin compatibility test using the isolated perfused bovine udder skin (7) was performed to compare the effects of the newly developed anti-louse product Wash-Away-Laus® and its pure, active ingredient, and chemicals used in the production process on skin in comparison with a high quality marketed shampoo (Nivea Silky Shine®) in order to determine possible risks regarding skin irritations.

■ Material and Method

BUS Model

The *in-vitro* isolated perfused bovine udder skin (BUS) model was originally developed for use in pharmaceutical research and is used in the cosmetic and chemical industries for testing transdermal penetration and irritation studies (8-11). Depending on the strength of the irritant, a diminished cell viability and an increased prostaglandin E_2 concentration in the skin were found. Quite recently the udder model was used for penetration studies of L-carnitin-L-tartrate, an active ingredient of a liposomal hair tonic to promote human hair growth (12).

The cow udders are obtained fresh from slaughter houses. After pre-treatment, they are cleaned carefully and shaved in the laboratory. The test substances are applied after about 1 hour of aerobic adaptation of metabolism by perfusion with oxygenated, warmed Tyrode's solution. The continuous perfusion keeps the mammary gland, including skin, viable for more than 8 hours, during which time the horny layer barrier and the skin metabolism remain active. Details of the experimental procedure are published elsewhere (7).

Other advantages include a large application surface (up to 600 cm^2/udder side), on which numerous product tests can be carried out comparatively and cost-efficiently. The hirsute skin on the side of the udder is histologically and functionally similar to human skin (13-14).

Material and application

The undiluted test substances **(Table 1)** were applied once in a modified rinse-off manner to the skin (4 g / 100 cm^2) of four udders (n=4) by the means of a glass spatula (standardized study design). After 15 minutes the skin was cleaned gently with tissue paper to remove any remaining material. Fifteen minutes later (i.e. 30 minutes after starting the application) the 1st punch biopsy (D=6mm; Stiefel, Germany) was taken. The 2nd and the 3rd whole skin biopsies were taken after 1,0 h and 5,0 h after starting the application in order to detect possible cellular reactions, cell irritancy (prostaglandin E_2 concentration, PGE_2) and cell damage (cytotoxicity, MTT test) to be traced biochemically. The skin samples from treated and untreated areas were stored temporarily at -20 °C. To assure the viability of the udder, including skin tissue and quality of the perfusion procedure, a 2nd control area was designated.

A modified MTT test (µg formazan /µg net weight) was subsequently carried out.

Untreated **1**:	(Control 1)
Untreated **2**:	(Control 2)
Sample **1**:	Wash-Away-Laus® (Market product, active neem seed extract, 10%)
Sample **2**:	Wash-Away-Laus® (Formulation without neem seed extract)
Sample **3**:	Nivea Silky Shine® (benchmark: market product; Art.nr. 81441, Serial nr. 623421902 high quality shampoo, BDF, Hamburg, Germany)
Sample **4**:	DBE-2 = Solvent, used for the production of the neem seed extract)
Sample **5**:	Neem seed (*Melia azadirachta*) extract (pure)

Table 1 Samples (treated areas) and untreated areas (control)

Only undamaged, active mitochondria are able to transform methyl-thiazol-tetrazolium into the water-insoluble formazan complex. The concentration of eicosanoids (ng PGE_2 / µg net weight) was determined in order to measure arachidonic acid metabolism activity in the skin tissue (Institute for Pharmacology, Toxicology and Pharmacy, Veterinary University of Hanover).

The reliability of the test data was confirmed by Analysis of Variance and a LSD test for multiple comparisons. The MTT values and the PGE_2-tissue concentration were determined for all the exposure periods. The p values were calculated by paired t tests. No adjustment for multiplicity was done, p values ≤ 0.01 are considered as statistically remarkable (significant).

The skin irritation potential of a product was also defined as a comparable numerical quantity by taking the MTT and PGE_2 measurement data, calculating the percentage deviation relative to the data of the untreated skin area (=100%) and using this to obtain a combined, weighted total score (score of untreated skin: 0.0). In order to be predictive for repeated skin contact in humans the score evaluation was combined with three different exposure periods.

The shampoo Wash-Away-Laus® – a medicinal remedy (class 1) produced by the university spin-off company Alpha-Biocare GmbH, Düsseldorf, Germany) which is based on a patented neem-seed extract (Alpha-Biocare GmbH, Düsseldorf) and fine shampoo components was shown to be highly active on head lice (15-16).

Besides aqua, the product contains the following compounds according INCI: Neem-Extract, Polyglyceryl-3-Caprate, Cocamidopropyl Betaine, Dimethyl Glutarate, Glycerin, Dimethyl Adipate, Lauryl Glucoside, Dicaprylyl Ether, Lauryl Alcohol, Benzyl Alcohol, Benzoic Acid, Dimethyl Succinate, Sorbic Acid. No parfum oil mixtures were used.

Results

Viability of the model

The viability of the perfused udder tissue was given, the data of the control areas

obtained varied within the historical range. There was no statistically significant difference between control I and control II at any exposure period (**Table 3**).

Skin compatibility (score evaluation)
On the basis of the results of studies using human volunteers and of epidemiological comparisons and market experience as well, it is possible to consider that, if a total score below 2.5 is obtained in the BUS model, no skin irritation will occur in human beings even after repeated skin contact. The total score of untreated skin is calculated as 0.0. For cosmetic formulations such as skin protection products applied once, the total

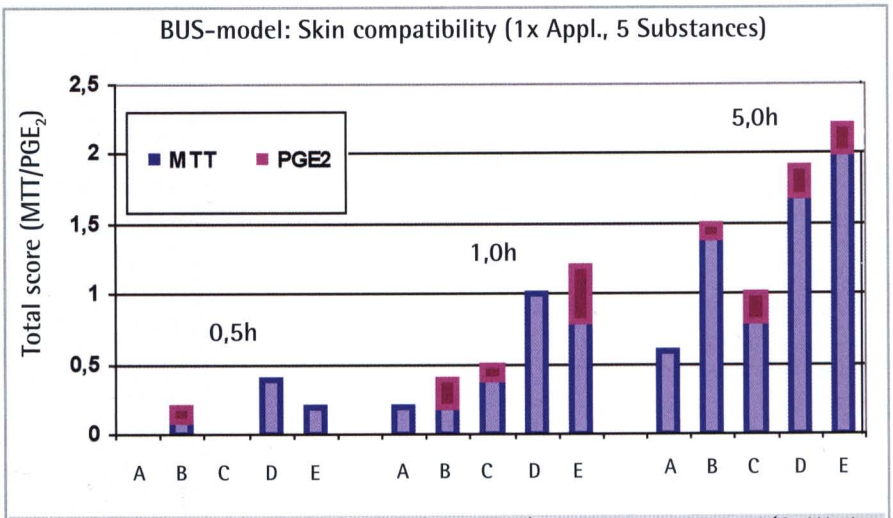

Table 2 Comparison of the score data (MTT, PGE_2) of all products tested (**A**=Wash-Away-Laus®, **B**=Nivea Silky Shine®, **C**=Wash-Away-Laus® without neem seed extract, **D**=solvent DBE-2, **E**=neem seed extract pure) after an exposure period of 0.5h, 1.0h and 5.0h and after one application

Comparisons	0.5h [n.s / s.]	1.0h [n.s / s.]	5.0h [n.s / s.]
MTT Control I vs Control II	[n.s.] 1.03 ± 0.10 vs 1.03 ± 0.09	[n.s.] 1.02 ± 0.10 vs 1.01 ± 0.08	[n.s.] 1.0 ± 0.10 vs 1.0 ± 0.09
PGE_2 Control I vs Control II	[n.s.] 0.53 ± 0.03 vs 0.52 ± 0.02	[n.s.] 0.54 ± 0.02 vs 0.52 ± 0.02	[n.s.] 0.52 ± 0.02 vs 0.52 ± 0.01
MTT Control I vs Wash-Away-Laus®	[n.s.] 1.03 ± 0.10 vs 1.00 ± 0.09	[n.s.] 1.02 ± 0.10 vs 0.96 ± 0.10	[s.] 1.0 ± 0.10 vs 0.92 ± 0.09
PGE_2 Control I vs Wash-Away-Laus®	[n.s.] 0.53 ± 0.03 vs 0.53 ± 0.02	[n.s.] 0.54 ± 0.02 vs 0.56 ± 0.01	[n.s.] 0.52 ± 0.02 vs 0.54 ± 0.02
MTT Control I vs Nivea Silky Shine®	[n.s.] 1.03 ± 0.10 vs 1.00 ± 0.10	[s.] 1.02 ± 0.10 vs 0.96 ± 0.09	[s.] 1.0 ± 0.10 vs 0.88 ± 0.08
PGE_2 Control I vs Nivea Silky Shine®	[n.s.] 0.53 ± 0.03 vs 0.54 ± 0.02	[n.s.] 0.54 ± 0.02 vs 0.58 ± 0.01	[n.s.] 0.52 ± 0.02 vs 0.54 ± 0.03
MTT Wash-Away-Laus® vs Nivea Silky Shine®	[n.s.] 1.00 ± 0.09 vs 1.00 ± 0.10	[n.s.] 0.96 ± 0.10 vs 0.96 ± 0.09	[n.s.] 0.92 ± 0.09 vs 0.88 ± 0.08
PGE_2 Wash-Away® vs Nivea Silky Shine®	[n.s.] 0.53 ± 0.02 vs 0.54 ± 0.02	[n.s.] 0.56 ± 0.01 vs 0.58 ± 0.01	[n.s.] 0.54 ± 0.02 vs 0.54 ± 0.03

Table 3 The statistical evaluation (mean ± sd; $p \leq 0.01$; s = significant, n.s. = not significant) of the control areas I and II and the treated skin samples concerning the parameter cytotoxicity (MTT; µg formazan / µg net weight) and irritancy (PGE_2, ng PGE_2 / µg net weight) at the three exposure periods (0.5h, 1.0h, 5.0h)

score ranges within 0.4 and 0.0 (17). Various skin care products produce a total score up to 1.0 at one of the three exposure periods using the standardized study design (unpublished results). With increasing exposure, cytotoxic cellular reactions are the main features observed **(Table 2)**. After the short exposure period (0.5h), almost no reaction occurred compared to the untreated skin areas. After the exposure period of 1.0h slight cytotoxic reactions occurred after DBE-2 or *neem seed extract (pure)* was applied. The application of Wash-Away-Laus®, Nivea Silky Shine® or the *basic solution without neem seed extract* induced no significant cellular reactions. However, higher cytotoxic and inflammatory reactions were induced by application of DBE-2 and *neem seed extract pure* after the prolonged exposure period of 5.0h. There is a small difference between the products Wash-Away® and Nivea Silky Shine®. The benchmark product elicited a slightly higher cytotoxic potential when compared with Wash-Away-Laus®.

Comparisons of products, active ingredient, chemicals (statistical evaluation)

Compared with the control area there is no difference between the two products Wash-Away® and Nivea Silky Shine® concerning the skin compatibility regarding cell irritancy (PGE_2). However concerning the cytotoxic reaction the application of Wash-Away-Laus® induced slightly less reaction (exposure period: 5.0h) than the application of Nivea Silky Shine® (exposure period: 1.0h and 5.0h) **(Table 3)**. Between the products Wash Away® and Nivea Silky shine® no statistically significant difference regarding skin compatibility can be considered if compared directly to each other **(Table 3)**.

Statistically significant deviations from the control were found most frequently after the application of the solvent (DB-2) and the active ingredient neem

seed extract (pure). After the short exposure period of 0.5h the application of all three test substances induced cytotoxicity and inflammatory reaction (DBE-2) throughout all exposure periods. The tissue concentration of PGE_2 also increased after exposure of 1.0h and 5.0h to the solvent **(Table 4)**.

Discussion

Heavily head louse infested skin shows exudation and itching at the bite sites, which may lead to localized infections. If the anti-louse product used has any capability for skin irritation, these minimal lesions may become sites of painful lesions. The efficacy of the shampoo Wash-Away-Laus® - a medicinal remedy class 1 - was tested in several clinical studies, and was shown to be highly effective on head lice (15-16). During these studies the probands declared that there is support of healing effects on infected louse bites without any burning **(Fig. 3)**. All test persons during efficacy tests had described that the use of Wash-Away-Laus® is very agreeable, since it smoothens the structure of the hair, has no painful effects at the bite sites and has a pleasant aroma. In dermatological tests in concentrations of 1%, 2% and 5% (Epicutaneous test, Project 46/05/07; Dermatest GmbH, Münster, Germany) the product proved to be very compatible on human skin. According to the test results, the rating was considered »very good«. Therefore, a carefully designed comparison concerning skin compatibility was performed in order to manifest scientifically the described »good feeling«.

Fig. 3 Volunteer study: Treatment of the scalp by pooring the shampoo

The basic product formulation of Wash-Away-Laus® and the benchmark product Nivea Silky Shine® are similar with the exception that Wash-Away® contains neem seed extract pure as the active ingredient and no parfume oil components. Therefore, it was of interest to compare the skin compatibility of both qualified hair care products. The application of these sort of products intended repeated human skin contact. Using the standardized method of the BUS model, a direct comparison is possible because the epidermal cellular reaction can be biochemically assessed from whole skin biopsies after various exposure periods. The primary exposure period exceeds the usual rinse-off time pe-

Comparisons	0.5h [n.s / s.]	1.0h [n.s / s.]	5.0h [n.s / s.]
MTT Control I vs Wash- Away-Laus® without NEEM	[s.] 1.03 ± 0.10 vs 0.99 ± 0.09	[n.s.] 1.02 ± 0.10 vs 0.95 ± 0.08	[s.] 1.0 ± 0.10 vs 0.91 ± 0.08
PGE_2 Control I vs Wash- Away-Laus® without NEEM	[n.s.] 0.53 ± 0.03 vs 0.53 ± 0.03	[n.s.] 0.54 ± 0.02 vs 0.56 ± 0.02	[n.s.] 0.52 ± 0.02 vs 0.53 ± 0.01
MTT Control I vs DBE-2 (solvent)	[s.] 1.03 ± 0.10 vs 0.96 ± 0.09	[s.] 1.02 ± 0.10 vs 0.92 ± 0.10	[s.] 1.0 ± 0.10 vs 0.87 ± 0.08
PGE_2 Control I vs DBE-2 (solvent)	[n.s.] 0.53 ± 0.03 vs 0.54 ± 0.02	[s.] 0.54 ± 0.02 vs 0.58 ± 0.01	[n.s.] 0.52 ± 0.02 vs 0.57 ± 0.00
MTT Control I vs Neem seed extract (pure)	[s.] 1.03 ± 0.10 vs 0.97 ± 0.09	[s.] 1.02 ± 0.10 vs 0.93 ± 0.08	[s.] 1.0 ± 0.10 vs 0.85 ± 0.08
PGE_2 Control I vs Neem seed extract (pure)	[n.s.] 0.53 ± 0.03 vs 0.52 ± 0.02	[n.s.] 0.54 ± 0.02 vs 0.58 ± 0.02	[s.] 0.52 ± 0.02 vs 0.55 ± 0.02

Table 4 The statistical evaluation (mean ± sd; $p \leq 0.001$; s = significant, n.s. = not significant) of the control areas and the treated skin samples (product formulation without NEEM, solvent, Neem pure) concerning the parameter cytotoxicity (MTT, µg formazan/µg net weight) and irritancy (PGE_2, ng PGE_2 / µg net weight) at the three exposure periods (0.5h, 1.0h, 5.0h)

riod, despite wiping away of the surplus after 15 minutes and all substances were applied undiluted. The application of DEB-2 and undiluted neem seed extract provoked the highest cytotoxicity and inflammatory reactions, increasing throughout the exposure periods. So both substances were biologically active within the epidermis and dermis, but no skin irritation can be predicted after repeated skin contact.

For both hair care products already marketed, the results of the score evaluation indicate excellent skin compatibility even after repeated skin contact. This result could be expected in testing a well known and widely used brand as a benchmark. The total score may be also directly compared with other cosmetic products such as skin care products and skin protection formulations (17). The results of the BUS-skin compatibility test confirmed the results obtained in human patch tests using Wash-Away-Laus®.

In addition to the score evaluation especially designed for the predictive results of repeated skin contacts, the biochemically assessed cellular reactions after a modified rinse-off situation can be used for further differentiation between the products. Despite the very slight differences of Wash-Away-Laus® and Nivea Silky Shine® in comparison to the untreated skin no statistically significant difference was observed in the direct comparison to each other. The degree of excellent skin compatibility is equal for both products even Wash-Away-Laus® a registered medicinal product, contains 'neem seed extract' as the biologically active ingredient.

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* *Correspondence address:*
Wolfgang Pittermann
Schwarzbachstrasse 33
40625 Düsseldorf
Email: dr.wolfgang.pittermann@gmx.at

** Walter Lehmacher
Institute for Medical Statistics, Informatics and Epidemiology
University of Cologne

*** Manfred Kietzmann
Institute for Pharmacology, Toxicology and Pharmacy
Veterinary University of Hanover

**** Heinz Mehlhorn
Institute for Zoomorphology, Cellbiology and Parasitology
Heinrich Heine University Düsseldorf