



## Penetration Experiment (Pro-Ferm).

One of the problems with non surgical cosmetic skin treatment is penetration. The surface of all skin is predominantly dead cells - to be efficacious any treatments need to penetrate to the epidermis.

Here the constituent elements of Pro-Ferm are tested on pig skin in an experiment that proves the exceptional penetration obtained by the creams (all treatment was post-mortem and the animal was not involved in or affected by the experiment).

Here are excerpts of the piece from **The Journal of Cosmetic Dermatology** - The Official Journal of the European Society for Cosmetic and Aesthetic Dermatology (ESCAD) - the full version can be downloaded as a pdf by [clicking here](#) or pages can be enlarged by clicking on them.

## Introduction

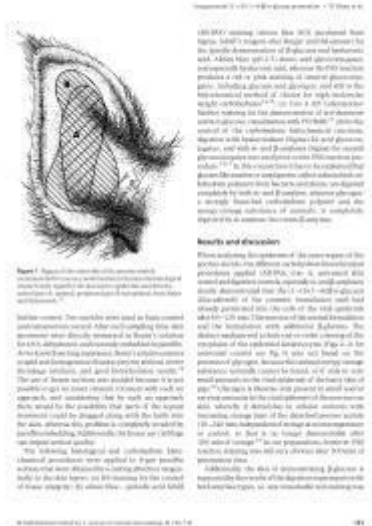


Bioartificial skin and artificial dermis, in particular, for wound dressing to mimic the normal human dermal skin is mainly composed of gelatine and polysaccharides such as hyaluronic acid and (1-3)-(1-6)-D-glucans.<sup>1,2</sup> The latter glycoconjugate group of multibranched glucose polymers is a nonallergenic structural cell wall component of most fungi or plant storage substances, and, concerning the skin, seems of great importance with regard to the water storing and rheological properties of artificial dermis.<sup>3</sup> It has also been reported that  $\beta$ -glucans, which have  $\beta(1-3)$  linkages in the main chain with additional  $\beta(1-6)$  branches, possess antimicrobial and antitumor activities by enhancing the most immune functions.<sup>4</sup> In our study,  $\beta$ -glucans represented one part of a cosmetic formulation, and we were interested in their permeation abilities, especially into and through the epidermis, as documented by our carbohydrate histochemical approach.

The experimental basis was porcine skin that has been of interest as a model for the human integument for more than 20 years, whereby the epidermis and the dermis show several structural and functional similarities to human skin as useful for the testing of transdermal systems, or the control of skin permeability.<sup>5-10</sup> Regarding the integument of the porcine auricle, it has been demonstrated that one region of the outer side can be of value in this connection.<sup>11-13</sup> Considering that pig keeping and handling is time-consuming and very expensive for routine testing, the use of porcine auricles from freshly dead animals for experimental purposes was recommended. If handled correctly, relevant scientific results can be achieved without difficulties.<sup>10</sup> This is, additionally, cost-efficient and saves laboratory animals.

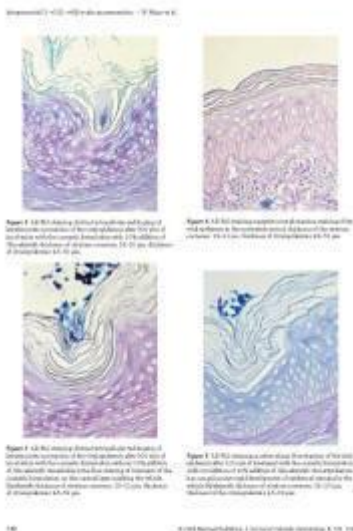
## Materials and methods

The auricles of five pigs (German Landrace/German Yorkshire; females, 6 months old, weight 110-120 kg) were obtained immediately after the animals had been sacrificed in the slaughterhouse, whereby the ears had not been scalded or flamed because such a pretreatment completely destroys the epidermis. One half of the marked part of the outer region (Fig. 1) of eight auricles was carefully covered for 30, 60, 120, and 300 min each with a cosmetic formulation [Pro-Cell3 Night Care, with Active Carrier System, containing 25-30% (1-3)(1-6) $\beta$ -d-glucans (MW 110,000 daltons,  $\beta$ -glucans of lower MW have not been found until now as far as we know) (Glucaferm®) and Na-hyaluronate (Hyaferm®); Fibona Health Products GmbH, Wiesbaden, Germany] without, and the other half with a surplus addition of 10% Glucaferm® (n = 4 for each time period, n = 16 for total formulation penetration); the latter aspect served as a further control. Two auricles were used as basic control and remained uncovered. After each sampling time, skin specimens were directly immersed in Bouin's solution for 48 h, dehydrated, and routinely embedded in paraffin. As we know from long experience, Bouin's solution ensures a rapid and homogeneous fixation process without severe shrinkage artefacts, and good histochemical results.<sup>14</sup> The use of frozen sections was avoided because it is not possible to get an intact stratum corneum with such an approach, and considering that by such an approach there would be the possibility that parts of the topical treatment could be dragged along with the knife into the skin, whereas this problem is completely avoided by paraffin embedding. Additionally, the frozen ear cartilage can impair section quality.



The following histological and carbohydrate histochemical procedures were applied to 8- $\mu$ m paraffin sections that were obtained by a cutting direction tangentially to the skin layers: (a) HE-staining for the control of tissue integrity; (b) alcian blue - periodic acid Schiff (AB-PAS) staining (alcian blue 8GX purchased from Sigma, Schiff's reagent after Barger and DeLamater) for the specific demonstration of  $\beta$ -glucans and hyaluronic acid. Alcian blue (pH2.5) stains acid glycoconjugates, and especially hyaluronic acid, whereas the PAS reaction produces a red or pink staining of neutral glycoconjugates, including glucans and glycogen, and still is the histochemical method of choice for high molecular weight carbohydrates<sup>14,15</sup>; (c) Con A (EY Laboratories/ Sanbio) staining for the demonstration of  $\alpha$ -d-mannose and  $\alpha$ -d-glucose, visualization with PO/DAB;<sup>16</sup> (d) for the control of the carbohydrate histochemical reactions, digestion with hyaluronidase (Sigma) for acid glycoconjugates, and with  $\alpha$ - and  $\beta$ -amylases (Sigma) for neutral glycoconjugates was used prior to the PAS reaction procedure.<sup>15,17</sup> In this connection it has to be explained that glucans like amylose or amylopectin, rather unbranched carbohydrate polymers from bacteria and plants, are digested completely by both  $\alpha$ - and  $\beta$ -amylase, whereas glycogen, a strongly branched carbohydrate polymer and the energy storage substance of animals, is completely digested by  $\alpha$ -amylase, but resists  $\beta$ -amylase.

## Results and discussion



When analysing the epidermis of the outer region of the porcine auricle, the different carbohydrate histochemical procedures applied (AB-PAS, Con A, untreated skin control and digestion controls, especially  $\alpha$ - and  $\beta$ -amylases) clearly demonstrated that the (1-3)(1-6) $\beta$ -d-glucans (Glucaferm®) of the cosmetic formulation used had already penetrated into the cells of the vital epidermis after 60-120 min. This was true of the normal formulation and the formulation with additional  $\beta$ -glucans. The distinct medium-red to dark-red or violet coloring of the cytoplasm of the epidermal keratinocytes (Figs 2-4; for untreated control see Fig. 5) was not based on the presence of glycogen, because this animal energy storage substance normally cannot be found, or if only in very small amounts in the vital epidermis of the hairy skin of pigs.<sup>16</sup> Glycogen is likewise only present in small and/or varying amounts in the vital epidermis of the porcine ear skin, whereby it diminishes in cellular contents with increasing storage time of the detached porcine auricle (30-240 min; independent of storage at room temperature or cooled), so that is no longer demonstrable after 300 min of storage.<sup>18</sup> In our preparations, however, PAS reaction staining was still very obvious after 300 min of penetration time. Additionally, the idea of demonstrating  $\beta$ -glucans is supported by the results of the digestion experiments with both amylase types, i.e. any remarkable red staining was not visible after digestion with  $\beta$ -amylase so that, if at all, only very few glycogen granules were present,<sup>17</sup> and the rather strong reaction intensities originated from  $\beta$ -

glucan penetration into the cells. This finding was corroborated by the negative or weak intracellular reaction for the lectin Con A, that in the hairy skin seems more prominent.<sup>16</sup> It should also be mentioned that, even considering the fact that the skin of the freshly obtained porcine auricle remains rather intact or vital for a certain time,<sup>10</sup> there are no enzymes present in the stratum corneum that could hydrolyse high molecular glycoconjugates,<sup>6,8,9,19</sup> and thus alter the standard treatment conditions before and during substance penetration.

The hyaluronic acid (Hyaferm®) as part of the cosmetic formulation used was stained by the AB (pH2.5; no positive staining after hyaluronidase control<sup>15,16,20</sup>) and could be confirmed in its function as vehicle for the β-glucans, whereby this view is corroborated by the observation that, independent of a strong blue to violet colour of remnants of the formulation lying on the stratum corneum of the porcine ear skin, after 30-60 min the vital epidermis and the dermis were distinctly tinged stronger blue (Fig. 5) than normally detectable for the different skin layers of the pig.<sup>16,18</sup>

## Conclusions

The specific carbohydrate histochemical methods used in the course of this study have demonstrated that (1-3)(1-6)β-d-glucans penetrated from a cosmetic formulation into the cells of the vital epidermis of porcine ear skin, obviously with the help of hyaluronic acid as the vehicle.

Such β-glucans may be important for the stabilization of osmotic conditions in the keratinocytes. Independent of this finding, the study has confirmed that the skin of freshly obtained detached porcine auricles can be helpful as a model for the human integument when permeation experiments are concerned.

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