Optical Clearing of Skin Using Flashlamp-Induced Enhancement of Epidermal Permeability

V.V. Tuchin,[1]*** G.B. Altshuler,**[2] **A.A. Gavrilova,**[1] **A.B. Pravdin,**[1] **D. Tabatadze,**[2]
J. Childs,[2] **and I.V. Yaroslavsky**[2]

[1]*Research-Educational Institute of Optics and Biophotonics, Saratov State University, Saratov, Russia*
[2]*Palomar Medical Technologies, Inc., Burlington, Massachusetts*

Background and Objectives: Strong light scattering in skin prevents precise targeting of optical energy in therapeutic and diagnostic applic ations. Optical immersion based on matching refractive index of scattering centers with that of surrounding matter through introduction of an exogenous index-matching agent can alleviate the problem. However, slow diffusion of the index-matching agent through skin barrier makes practical implementation of this approach difficult. We propose a method of accelerating penetration of the index-matching compounds by enhancing skin permeability through creating a lattice of micro-zones (islets) of limited thermal damage in the stratum corneum (SC).
Study Design/Materials and Methods: A flashlamp (intense pulsed light) system and an island mask with a pattern of absorbing centers (center size $\sim$75–120 μm, lattice pitch $\sim$450–500 μm) were used to create the lattice of islets of damage (LID). Index-matching agents, such as glucose solution, propylene glycol solution, and glycerol solution, were applied.
Results: Experimental results of optical clearing ex vivo rat and pig skin, and ex vivo and in vivo human skin are presented. Optical transmission spectra of the skin samples with LID were measured during some 2 hours after application of index-matching chemical agents. In order to assess and compare the clearing rate under different treatment and clearing agents we calculated the quantity that we call "relative transmittance": $T_{rel} = I_t(\lambda)/I_0(\lambda)$, were $I_t(\lambda)$ is the intensity measured at elapsed time t. The dynamics of relative transmittance of skin samples at 470 and 650 nm shows that the implementation of limited thermal damage technique leads to a 3–10-fold increase of optical clearing (rise of transmittance) rate compared to the results obtained when the samples were treated with high-intensity light pulses but without the use of island damage mask (IDM). It was observed from the plotted spectra of relative transmittance that the maximum increase of transmitted light intensity has been obtained with glucose solution as a clearing agent. Noteworthy is the difference in the trend of spectral curves: relative transmittance spectrum for glycerol reveals, on the whole, a greater slope which may be indicative of higher extent of index matching between the scattering centers and base material for this index-matching agent. Under the transillumination of the skin sample by the wide flat beam the more effective clearing (the increase of transmitted intensity) is attained within the hemoglobin absorption bands; with the narrow quasi-collimated beam the higher relative transmittance was observed over the intervals of minimum absorption.
Conclusions: The use of specially designed island mask combined with non-laser intensive pulse irradiation produces a lattice of islands of limited thermal damage in SC that substantially enhances the penetration rateof topically applied index-matching agents. The suggested technique gave comparable magnitudes of clearing dynamics enhancement for glucose solution, glycerol solution, and propylene glycol solution applied to mammalian skin. Lasers Surg. Med. 38:824–836, 2006. © 2006 Wiley-Liss, Inc.

INTRODUCTION

Nowadays we are witnessing the overgrowing interest to the field of biomedical optics that some 10 years ago seemed rather exotic-controlling of optical properties of biological tissues [1–7].

The vast majority of tissues are known to be low absorbing but highly scattering media in the visible and near infrared spectral regions. Scattering defines spectral and angular characteristics of light interacting with living objects, as well as its penetration depth; thus, optical properties of living tissues may be effectively controlled by alteration of its scattering properties. At longer wavelengths (approximately longer than 900 nm), water absorption starts to play a substantial role in light attenuation. Thus, additional facilitation of light propagation can be effected in this spectral region if water content of tissue is decreased. The physico-chemical organization and nature of tissue allows one to alter its optical (scattering)

properties using various physical and chemical factors such as compression, stretching, dehydration, coagulation, UV irradiation, exposure to low temperature, and impregnation by chemical solutions, gels, and oils [6]. The multiplicity of processes underlying these effects can be understood if we consider tissue as a scattering medium that shows all optical peculiarities characteristic to turbid physical systems. It is well known that the matching of refractive indices of scatterers and ground material can effectively control turbidity of a dispersion medium. This forms a basis of so-called optical immersion technique to control tissue optical properties.

Skin often is the first organ to face incident probing or treating light beam, and strong light scattering within this tissue prevents precise targeting of optical energy in therapeutic and diagnostic applications. Optical immersion based on introduction of an exogenous index-matching substance or optical clearing agent (OCA), can alleviate the problem. A number of laser surgery, therapy, and non-invasive diagnostic technologies may have a significant benefit at a reversible scattering reduction. Such biocompatible OCAs can be glucose, mannitol, propylene glycol, glycerol, polyethylene glycol, polypropylene glycol, X-ray contrasting agents (Verografin, Trazograph, and Hypaque), demethylsulfoxide (DMSO), and their combinations. Experimental studies on optical clearing of normal and pathological skin and its components (epidermis and dermis), and the control of skin reflectance and transmittance spectra using water, glycerol, glycerol-water solutions, glucose-water solutions, sunscreen creams, cosmetic lotions, gels, and pharmaceutical products have been carried out [6,8–18]. The control of skin optical properties was related to the matching of refractive indices of scatterers (keratinocyte components in epidermis, collagen, and elastic fibers in dermis) and ground matter induced by OCA permeation and/or tissue dehydration due to the osmotic properties of OCA [8–18].

A variety of chemical agents with different optical clearing potential (OCP), defined as the ratio of values of tissue reduced scattering coefficient before and after agent action, $OCP \equiv \mu_s'$ (before)$/\mu_s'$ (after) was recently studied [17]. OCP was measured in vitro at agent application to dermis side of human skin using a Franz diffusion chamber after 20 minutes application time. It was found no correlation between OCP and refractive index for used agents with indices in the range from 1.43 to 1.48, as well as no correlation with osmolality in a wide range from 1,643 to 26,900 mOsm/kg. However, the highest values of OCP from 2.4 to 2.9 were provided by the agents having both the highest refractive index and osmolality, such as glycerol, 1,4-butanediol, and 1,3-butanediol.

However, slow diffusion of OCAs through human skin barrier makes practical application of optical immersion effect difficult. To reduce barrier function of the skin a number of different chemical and physical methods were proposed [19–30]. For example, approximately 100-fold increase in permeability of polar molecules, such as urea, mannitol, sucrose, and raffinose for ethanol pretreated human epidermal membrane (HEM) relative to intact HEM is explained by increased porosity of HEM at extraction of HEM lipids by ethanol [21]. Effects of ethanol/propylene glycol composition on macroscopic barrier properties of the skin with respect to aspirin transport were also described [22]. Besides ethanol, a number of chemical agents may serve as enhancers of cell membrane permeation [19,20]. For example such polyenic antibiotic as Amphotericin B provides a twofold increase of water permeation through a cell membrane, more than 44-fold for glycerol and more than 200-fold for urea [20]. Dimethylsulfoxide (DMSO), a polar aprotic solvent, is also a good enhancer. A concentration of approximately 60% is required for activity of DMSO to disrupt human skin barrier function; and enhancement ratios of 20–200 have been reported [19]. The monounsaturated fatty acid, oleic acid (C18), is frequently chosen as an enhancer for a wide variety of polar and moderately lipophilic compounds [19,25].

Stripping of the stratum corneum (SC) represents the simplest approach to physical enhancement of the percutaneous absorption of a topically applied compound [9,19,26]. Ultrasound technique (phonophoresis or sonophoresis) provides the enhanced skin absorption of low-molecular-weight compounds as well as proteins such as insulin [19]. In vivo application of ultrasound of 20 kHz to skin of hairless rats for 1 hour resulted in a 100-fold increase in transepidermal water loss and sufficient delivery of insulin through the skin. Iontophoresis refers to the enhancement of agent percutaneous absorption by the application of moderate (0.5 V/cm) voltages across the skin [9,19]. At higher voltages (5–200 V/cm) and short pulse exposure electroporation of biological membranes may occur, that also enhances agent permeation into a tissue.

It was shown recently that laser-generated stress waves (photomechanical waves) can also permeabilize the SC [27]. This effect was first demonstrated with δ-aminolevulenic acid (ALA) as a probe. The application of stress waves does not cause any pain and discomfort and does not appear to affect the structure and viability of the skin. The change of the permeability of the SC is transient and its barrier function recovers within a few minutes. The increased permeability allows macromolecules to diffuse through the SC to epidermis and dermis [27]. The combined action of laser-stress waves and anionic surfactant, such as sodium lauryl sulfate (2% of w/v), enhanced the delivery of nanoparticles through the SC [27].

Alternative techniques of clearing agent delivery based on injection of an agent into the skin with needle-free injection gun and laser skin surface ablation, and their combinations are also under development [14,28–30]. The SC ablation can be provided directly by application of the pulsed Erbium lasers with the wavelengths of 2,790–2,940 nm corresponding to the strong water absorption band [28,29]. Among such modalities, as skin microdermabrasion, iontophoreses, electroporation, and Erb:YAG ($\lambda = 2940$ nm) ablation, tested by the authors of Reference [28], laser ablation showed the greatest enhancement of ALA permeation through the pig skin samples. The laser

fluence was found to play an important role in controlling the drug flux, producing enhancement ratios up to 246-fold relative to the control. Laser ablation of 12.6% of the porcine SC surface as a number of 2-mm-diameter ablated areas produced a 2.8- and 2.1-fold increase in permeability constant (P_a) for ^{3}H-hydrocortisone and ^{125}I-γ-interferon, respectively [29]. The similar increase of skin permeability was obtained for the tape stripped SC. These studies demonstrate that a 250 microseconds-pulsed laser with the wavelength of 2,790 nm and 1 J/cm^2 fluence can reliably and precisely remove the SC at 10–14 laser pulses, facilitating penetration of large molecules such as ^{125}I-γ-interferon that cannot penetrate intact skin.

A diode laser source with 980 nm wavelength in conjunction with an artificial absorber on the skin surface was used to facilitate enhanced penetration of the topically applied skin clearing agent glycerol into in vivo hamster and rat skin [30]. Such technique provides a sufficient skin surface heating, which leads to keratinocyte disruption and possibly skin surface ablation of less than 20 μm.

Recently, we proposed a method of accelerating penetration of the OCAs by enhancing epidermal permeability via creating a lattice of micro-zones (islets) of limited photothermal damage or lattice of islets of damage (LID), in the SC. It is anticipated that the restructuring and/or ablation of SC under photothermal damage would enhance transepidermal drug penetration, OCA being among the possible penetrating species. As the damage of the SC is not a damage of viable tissue, long-term effect of such damage may be only the transient deterioration of skin barrier function. That might lead to the local increase of transdermal water losses and the elevated sensitivity to irritants as possible site-effect. In our extended in vivo studies (not published yet), when we performed some 50 LID treatments, the primary erythema after photothermal ablation disappears during 24 hours, and the subsequent 1-week observation did not reveal any late effect (irritation, inflammation, pigmentation, discomfort, etc.).

Creation of a lattice of localized areas of light–tissue interaction (optical islets) is an example of the "spatially confined" approach. The lattice of optical islets can be formed using a variety of energy sources and delivery optics, including application of lenslet arrays, phase masks, and matrices of exogenous chromophores [31]. In practical implementation of the method we exploited the last-mentioned option—application of a specially designed appliqué with a pattern of absorbing centers with the following irradiation by flashlamp pulses. After application of the OCAs (glucose solution and glycerol solution) to the rat skin subjected to such procedure in vitro, we observed the marked increase of sample translucence [32].

The objective of this article is the experimental assessment of feasibility of the LID technique to accelerate the process of skin optical immersion clearing via the enhancement of *stratum corneum* permeability. We present the experimental results on optical clearing of ex vivo rat and pig skin and ex vivo human skin. Optical transmission spectra were measured after the application of OCAs on the LID-treated sample.

MATERIALS AND METHODS

Skin Samples

Experiments were performed with the fresh rat skin samples ex vivo, farm pig and Yucatan pig skin samples ex vivo, human skin samples ex vivo, and human skin in vivo. Full-thickness pig skin was excised from the abdomen region of a 1.5-month male pig. Subcutaneous fat was removed and some samples of pig skin were gently shaved before experiments. The thickness of pig skin samples was about 1 mm. Full-thickness rat skin was excised from the dorsal region of adult albino laboratory rat. Subcutaneous layer was removed from samples. The hairs of rat skin were carefully and gently plucked up before experiments.

We used an intact human skin samples taken from a cadaver 4 hours after death. The sheet of human skin was stored in physiological solution before measurements. The skin sample consisting of epidermis and dermis was prepared by removing the subcutaneous tissue with blunt scissors. The resulting samples were cut into 2×2 cm clouts with a 2 mm thickness. Epidermal surface of the samples was cleaned with alcohol to remove possible contamination by subcutaneous fat and air dried.

Experiments with human skin in vivo were carried out on the skin of a healthy male volunteer (a dorsum of the second phalanx of the fifth finger).

Chemical Agents

We performed our measurements with the biologically compatible index-matching agents most extensively used in experiments on optical clearing, namely: 100%-glycerol, 60% per volume glycerol solution in distilled water, 40% per weight glucose solution in distilled water, and 60% per volume propylene glycol solution in distilled water. As a marker for the indication of the transepidermal diffusion *loci* the dye methylene blue at the concentration of 0.65 mg/ml in 40% w/w solution of glucose was used.

Island Damage Mask

A specially designed appliqué with a pattern of carbon-black absorbing centers (center size ~75–120 μm, lattice pitch ~450–500 μm) has been used to create the LID. The dots (absorbing centers) were printed using a conventional laser printer on transparent plastic film. The use of the lattice of absorbing centers contacting with the very surface of SC is necessary for the localization of light absorption (during the photothermal treatment), and thus the thermal damage, within the thin surface layer, otherwise the heat generation would be spread over nearly the entire skin thickness. Microscopic image of the appliqué pattern of absorbing centers is shown in Figure 1. Size of the island damage mask (IDM) was 1×2 cm and corresponded to a handpiece window of the flashlamp system used.

Flashlamp System

A flashlamp (intense pulsed light) system (EsteLux or MediLux, Palomar Medical Technologies, Inc., Burlington, MA) was used. [31,32] The Palomar EsteLux System is designed to deliver light pulses (10–100 milliseconds) of
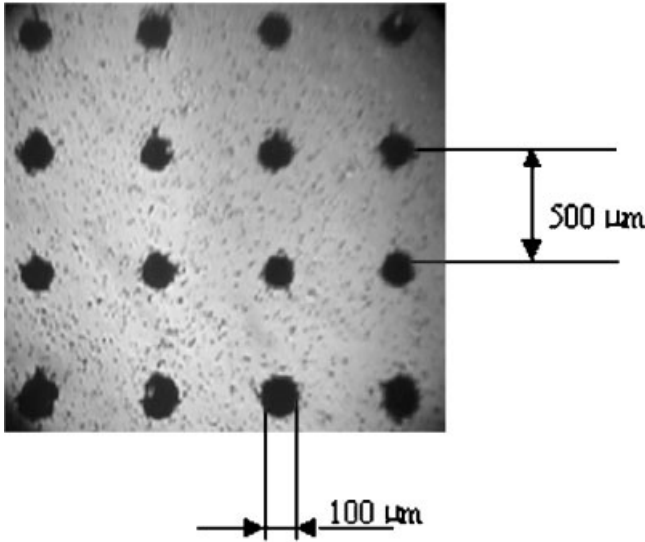
Fig. 1. Microscopic photography of island damage mask.

broadband incoherent light to a predetermined target site. Output wavelengths range from 400 to 1,400 nm. This system is specially developed for hair removal, pigmented, and vascular lesion treatment and other cosmetic application. The Palomar EsteLux and MediLux Systems allow the treatment, provider to select output fluence (J/cm^2) and pulse width (milliseconds) using the bottoms on the control panel of the system in accordance with the appropriate handpiece fluence chart. The handpiece is a cooled flashlamp housing connected by umbilical with the console of the system. The user holds the handpiece by the handpiece body during treatment. Delivery of light from the flashlamp is through a handpiece sapphire window. Light is administered to the treatment area by placing the handpiece window in contact with the skin. LuxRs (650–1,200 nm, window 12×28 mm, 9–27 J/cm^2), LuxY (525–1,200 nm, window 16×46-mm, 6–14 J/cm^2), and LuxB (470–1,400 nm, window 12×12-mm, 14–40 J/cm^2) handpieces were used in our experiments. Output fluence was 14, 20, 27, 36 J/cm^2, pulse width was 20 and 10 milliseconds.

Technique of LID and OCA Treatment

In the series of spectrophotometric experiments before the optical transmittance measurements either fresh pig skin samples ex vivo or human skin samples ex vivo were subjected to successive LID- and OCA treatment. Phototreatment was performed on the samples disposed on the flat hard surface with epidermis up. The IDM shown in Figure 1 was laid on the specimen with carbon-black centers down. The sapphire window of LuxRs handpiece was gently pressed against the IDM, and the sample received a certain number of 20 milliseconds light pulses in conditions of intimate contact. The excessive mechanical pressure was avoided to reduce the risk of possible pressure-induced clearing of the tissue. In some experiments, the transparent plastic film of IDM was removed from the surface of the sample, but the island pattern remained on the skin; and this skin site was repeatedly illuminated by a certain number of pulses. Before spectroscopic measurements the carbon-black particles, if still remained in the islets of thermal damage, were removed with alcohol. Then the phototreated sample was placed dermal surface down to a special holder for transmittance measurements, where it was treated by OCAs. The immersion agent was applied to the sample epidermal surface as a glittering liquid film. Only the central part of the skin clout was treated with solution in order to prevent the contact of OCA with the cut surface of the sample. During measurements the treated surface of the sample was kept wet by addition of fresh portions of OCAs. Specimen was heated up to about $50 \pm 2°C$ and remained under this elevated temperature during measurements. The heating of skin samples was provided by the use of a hairdryer (see Fig. 2). The temperature of the sample was measured using a thermometer (see Fig. 2). No special effort to control the temperature was made; and 50°C is the stationary mean value under the conditions of experiment.

Transmittance Measurements

Figure 2 shows the schematic and photograph of the system used in this study. As an illumination source the halogen lamp unit equipped with a fiber-optic bundle was used. Incident light spot on the skin sample surface had a diameter of 15 mm. In the restricted number of measurements we used a long-focus lens to form a narrow (2 mm-diameter) quasi-collimated beam coaxial with collecting fiber-optic probe. This probe consisting of six 200-µm fibers collected transmitted light from 2 mm-diameter area on the back surface of the sample within the collection angle of 25°. At the opposite end of the fiber-optic bundle the tips of the fibers formed a linear structure that served as an entrance slit of a holographic-grating polychromator LESA-7med (BioSpec, Russia) controlled by PC.

Spectral range of registration was 380–1,000 nm. Acquisition time of optical multichannel analyzer was set to 50 and 100 milliseconds for pig skin and 300 milliseconds for human skin. Spectral resolution of the instrument was
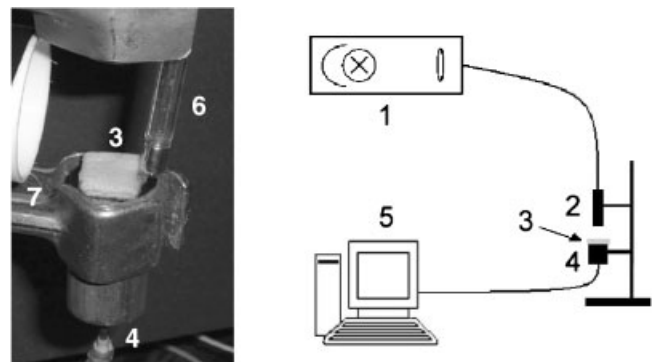

Fig. 2. Experimental setup: 1—halogen lamp unit, 2—fiber-optical bundle, 3—sample, 4—sample holder with mounted fiber-optic detector, 5—PC; 6—thermometer; 7—hairdryer hose.

8 nm. Readings of transmittance spectra were made every 5 minutes in duration of 1–1.5 hours of clearing process.

RESULTS AND DISCUSSION

Observation of Epidermal Lattice of Damage Islets and Enhanced Skin Optical Clearing Rate

In the first series of experiments we tested on human skin in vivo the feasibility of the use of suggested IDM for the formation of the lattice of limited thermal damage islets in epidermis. A dorsum of second phalanx of the fifth finger of healthy volunteer was cleaned with alcohol before treatment. Then IDM was placed on the alcohol-cleaned area, and skin in the projection of mask received two 20-milliseconds pulses, 14 J/cm^2 each, under the condition of intimate contact between skin, mask, and sapphire window of the handpiece. Pulse fluence of 14 J/cm^2 was chosen just near the pain threshold. After the exposure, the holder-film of the mask was removed and the surface of skin was cleaned with alcohol in order to remove black remains of absorbing centers. Polarized light photograph (parallel polarizers) clearly shows a lattice of thermal damage islets in epidermis (Fig. 3). Non-affected epidermis between islets looks glittering for surface reflection characteristic for normal epidermis. Zones of damage looks like ellipsoidal concavities.

In order to prove that formed damage islets serve as centers of enhanced permeability of epidermis to hydrophilic agents, the glucose solution with methylene blue added was applied to the treated skin area with LID and covered with a plastic bandage for approximately 30 minutes. Then the skin surface was rinsed with physiological solution. In Figure 4 the polarized light photograph of methylene blue treated skin area with LID is shown. In order to contrast the staining of the skin subsurface layers, crossed orientation of polarizes was used. In the photograph the pattern of centers of staining
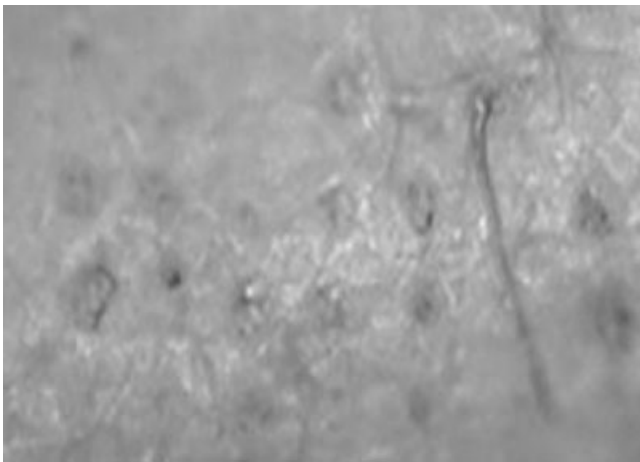

Fig. 3. Lattice of islets of damage in human skin in vivo after IDM+flashlamp treatment (two 20-milliseconds pulses, 14 J/cm^2 each). Polarization photograph with parallel polarizers.
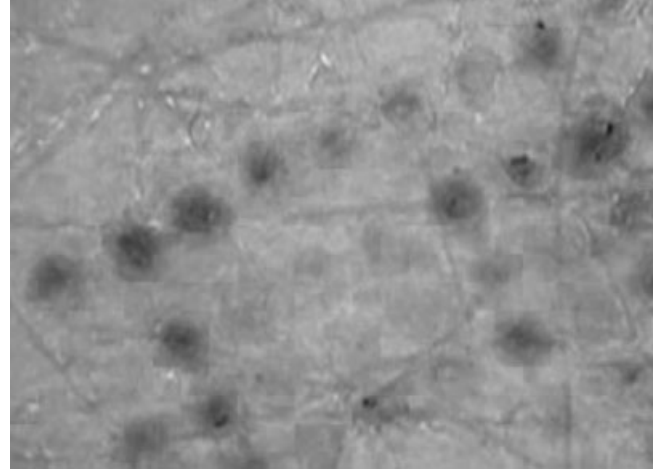

Fig. 4. Lattice of staining centers in human skin in vivo after IDM+flashlamp treatment (two 20-milliseconds pulses, 14 J/cm^2 each) followed by the topical application of glucose solution with methylene blue added. Polarization photograph with crossed polarizers.

(centers of the enhanced diffusion of MB into the tissue) can be easily seen to reproduce the lattice of islet damage. The non-uniformity in size and intensity of colored spots may be caused by the non-uniformity in size of absorbing centers of mask and non-uniformity of skin-mask contact under illumination for the anatomical peculiarities of underlying structures.

In the next part of the study, a piece of farm pig skin of approximately 4 cm^2 was glued on the rigid transparent film and cleaned with alcohol wipe. Dry skin surface was divided into four areas. IDM was placed on one of the areas and covered with a thin layer of Lux lotion. Two 1-cm^2 specimens had received two 20-milliseconds pulses of 36 J/cm^2 fluence each. One 1-cm^2 specimen had received two 10-milliseconds pulse of 20 J/cm^2 fluence. Last (fourth) none-treated area of specimen served as a control. Distances between the treated areas were about 1 cm. Thin blue wires were placed under the all studied areas of the specimen. Then the specimen was covered with 40% glucose solution and kept worm by using of hair dryer. Surface of sample was kept wet by addition of fresh portions of glucose solution. Optical clearing was assessed by observation of visual appearance of blue wires through the specimen.

Island damage procedure described above created hardly noticeable island damages on SC of farm pig skin specimen (Fig. 5). Maximum optical clearing was observed after 60 minutes OCA action for 36 J/cm^2—20-milliseconds light pulse. Application of 20 J/cm^2—10-milliseconds pulse gave a less pronounced clearing then 36 J/cm^2—20-milliseconds pulse irradiation. No detectable clearing was observed on the control (none-treated) area of specimen (Fig. 6).

In the experiments on rat skin ex vivo two specimens were divided into two areas each. Both areas were covered with OCA solution then the IDM was placed on only one part of the sample. In this section of the study we used 40%
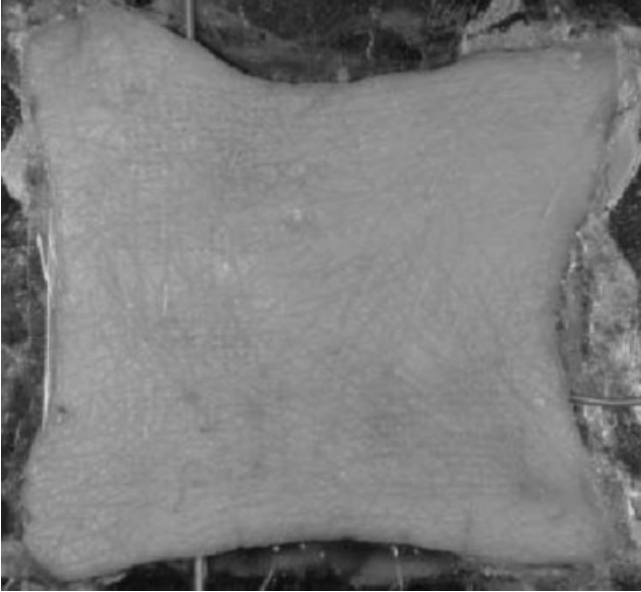
Fig. 5. LID-treated farm pig skin specimen glued on the rigid transparent film. There are hardly noticeable island damages on the SC of the specimen.

glucose solution on one sample and 100% glycerol on another. Glycerol has a refractive index of 1.46 and 40%-glucose solution has a refractive index of 1.39. Sheets of paper with printed letter "O" or the word "clearing" were placed under all four studied areas of the specimens. Prepared areas of rat skin specimens received two 20-milliseconds pulses of 14 J/cm^2 from LuxY handpiece of a flashlamp system. After the phototreatment the samples were photographed periodically for 140 minutes.

Photography was used to estimate the degree of optical clearing effect after the topical application of chemical agents and LID treatment by observation of visual
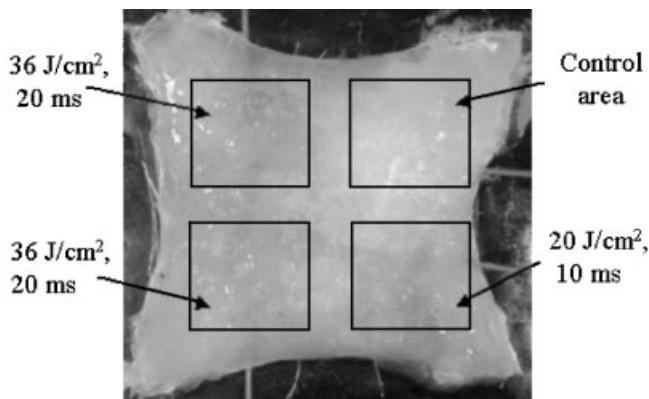

Fig. 6. Glucose-treated farm pig skin specimen, 60 minutes after OCA application. Maximum optical clearing was observed for 36 J/cm^2 (20 milliseconds). No detectable clearing was observed on the control (none-treated) area.
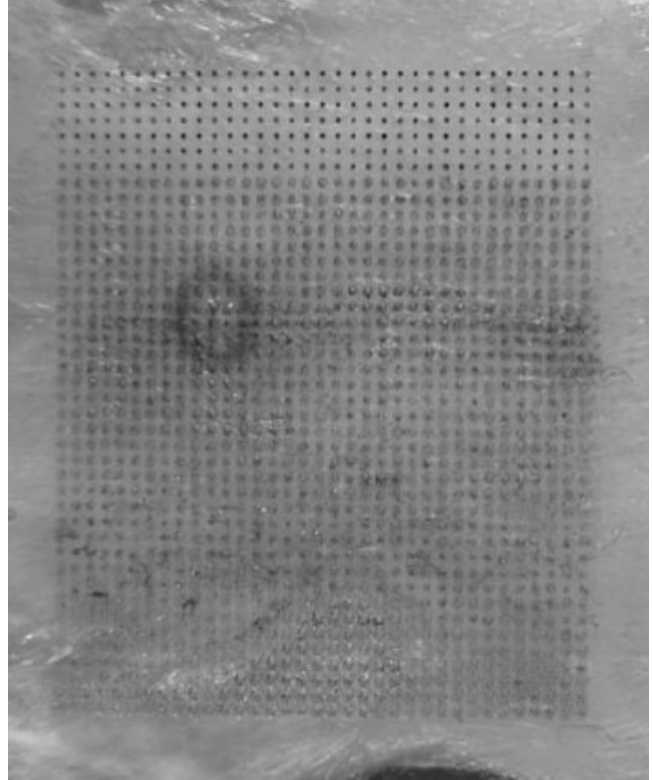

Fig. 7. Photograph of the rat skin sample 60 minutes after the treatment with glycerol and two light pulses (14 J/cm^2, 20 milliseconds) using IDM.

appearance of letter "O" or the word "clearing" through the specimens.

Photographs obtained in these experiments are presented in Figures 7–10. Pronounced optical clearing of rat skin in vitro was demonstrated for glycerol with LID created by IDM application+two optical pulses (fluence 14 J/cm^2, 20-milliseconds pulse). Maximal clearing effect was reached by the 60th minutes. Only a slight clearing was observed at the control site (treated with glycerol but not LID treated).

Optical clearing, but less pronounced, was also demonstrated for 40% glucose solution with LID created by IDM application+two optical pulses (fluence 14 J/cm^2, 20-milliseconds pulse). Only a slight clearing effect was observed at the control site (treated with glucose but not LID treated).

Spectrophotometric Results

In this part of study, the fresh pig skin samples and human skin samples ex vivo were used. Forty percentglucose water solution, 60%-propylene glycol water solution, and 60%-glycerol water solution were used as OCAs.

IDM was placed on the pig skin sample or human skin sample, and the sample received certain number of 20-milliseconds pulses of 14 J/cm^2 from LuxRs handpiece of the flashlamp system. Then sample was cleaned with alcohol and placed (dermal surface down) on the special
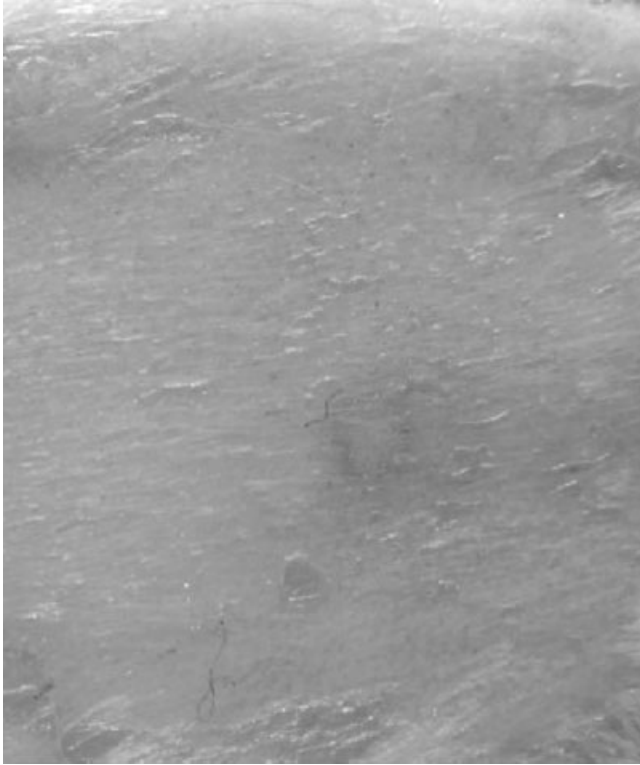
Fig. 8. Photograph of the rat skin sample 60 minutes after the treatment with glycerol and two light pulses (14 J/cm^2, 20 milliseconds) without IDM.

holder with mounted distal end of input fiber-optic bundle of the polychromator. Samples treated by LuxRs handpiece of a flashlamp system without IDM and the samples not subjected to phototreatment were used as controls. Immediately after the application of a solution of clearing agent, the hair dryer was switched on, and the first recording of the transmitted light spectrum, $I_0(\lambda)$, was performed. Then recordings were repeated at 5 minutes intervals. Through-
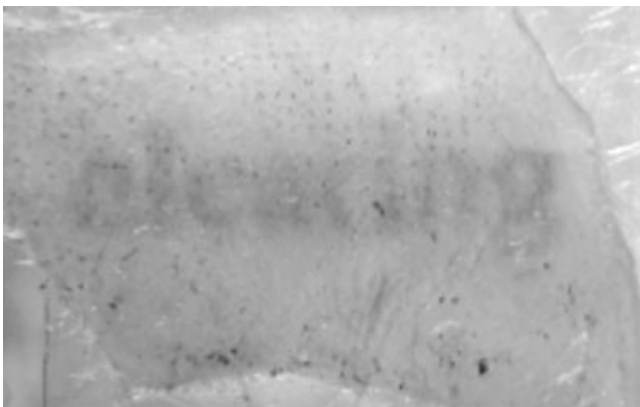

Fig. 9. Photograph of the rat skin sample 60 minutes after the treatment with 40%-glucose solution and two light pulses (14 J/cm^2, 20 milliseconds) using IDM.
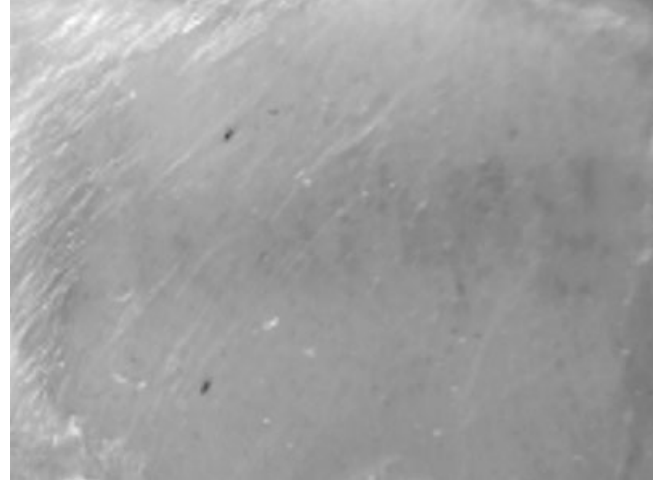

Fig. 10. Photograph of the rat skin sample 60 minutes after the treatment with 40%-glucose solution and two light pulses (14 J/cm^2, 20 milliseconds) without IDM.

out the measurements the surface of the sample was kept wet by adding of fresh portions of clearing agent. In order to assess and compare the clearing rate under different treatment and clearing agents we calculated, from the temporal measurements of transmitted light intensity, the quantity that we call "relative transmittance"

$$T_{rel} = \frac{I_t(\lambda)}{I_0(\lambda)} \tag{1}$$

were $I_t(\lambda)$ is the intensity measured at elapsed time t.

The dynamics of relative transmittance of skin samples at 470 and 650 nm is presented in Figures 11−15.

The data presented in the above figures are indicative that the OCA (water solutions of glucose, glycerol, and propylene glycol) topically applied on the intact epidermis, during the observation time exceeding 1 hour, did not produce a detectible increase of light transmission by the samples of human and pig skin. The treatment of the epidermal surface with high-intensity light pulses but without the use of IDM allowed us to record the occurrence of optical clearing, but the noticeable increase of clearing rate, for all the OCAs used, was attained only with the use of IDM. On average, the implementation of limited thermal damage technique leads to a 3−10-fold increase of optical clearing (rise of transmittance) rate compared to the results obtained without IDM. From the behavior of the curves 2 and 3 in Figure 12 which relate to two different irradiation doses it can be speculated that under the conditions of our measurements the increase of irradiation dose in LID-treatment correlates with the more pronounced tissue optical clearing (enhancement of light penetration).

As it can be seen from the presented transmittance dynamics at 470 and 650 nm, the clearing effect notably differs at different wavelengths. To compare clearing rate in different spectral regions we have plotted spectra of relative transmittance (see Figs. 16−18). Such spectra
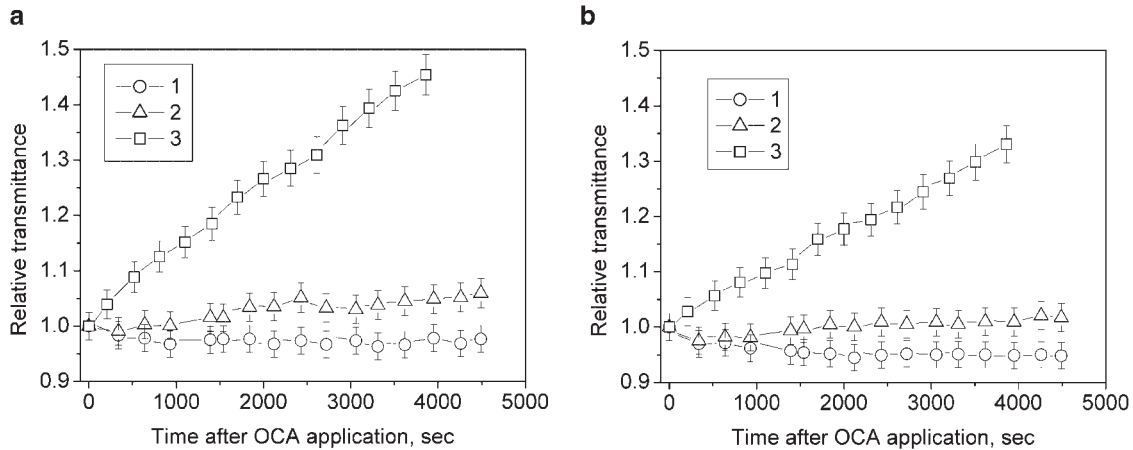
Fig. 11. The effect of LID treatment on the rate of the immersion clearing of pig skin ex vivo by 40%-glucose solution: 1—the control (no phototreatment); 2—control specimen received four 20-milliseconds pulses of 27 J/cm^2 without IDM; 3—specimen received two 20 milliseconds-pulses of 27 J/cm^2 with IDM and two 20-milliseconds pulses of 27 J/cm^2 after the removal of IDM. Results are presented for the wavelength of 470 nm (**a**) and 650 nm (**b**).

calculated using Equation (1) for all clearing agents used and 1-hour elapsed time are shown in Figure 16. It can be easily seen from the graph that the best results (maximum increase of transmitted light intensity) have been obtained with glucose solution as a clearing agent. However, these particular results hardly may be used in comparison of clearing efficacy of the agents, since in a case of experiment with glucose the applied IDM was irradiated by two light pulses, in other cases—by one pulse, thus the degree of SC damage and the conditions of OCA/water diffusion were not identical. Nevertheless, noteworthy is the difference in the trend of spectral curves: relative transmittance
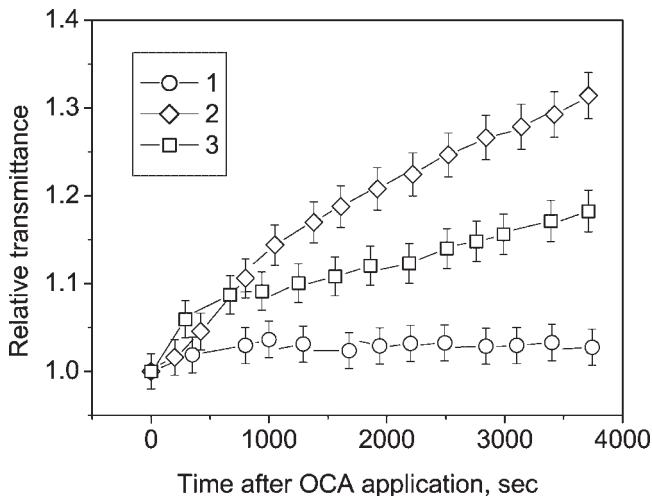

Fig. 12. The effect of the dose of LID-phototreatment on the rate of the immersion clearing of pig skin ex vivo by 40%-glucose solution (in contrast to other experiments, the sample in transmittance measurements was illuminated with a narrow quasi-collimated beam): 1—the control (no phototreatment); 2—specimen received one 20-milliseconds pulse of 27 J/cm^2 with IDM and six subsequent 20-milliseconds pulses of 27 J/cm^2 each after the removal of IDM; 3—specimen received one 20-milliseconds pulse of 27 J/cm^2 with IDM and two subsequent 20-milliseconds pulses of 27 J/cm^2 each after the removal of IDM. Results are presented for the wavelength of 650 nm.
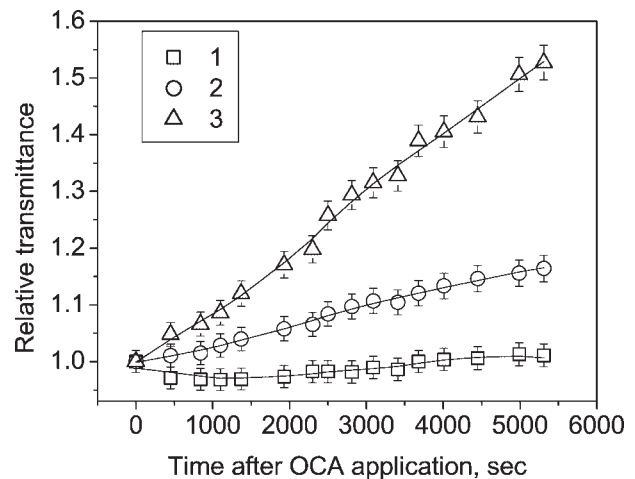

Fig. 13. The effect of LID treatment on the rate of the immersion clearing of human skin ex vivo by 40%-glucose solution at the different wavelengths of observation: 1—control specimen received three 20-milliseconds pulses of 27 J/cm^2 without IDM; 2—specimen received one 20-milliseconds pulse of 27 J/cm^2 with IDM and two 20 milliseconds-pulse of 27 J/cm^2 after the removal of IDM, 650 nm; 3—specimen received one 20-milliseconds pulse of 27 J/cm^2 with IDM and two 20-milliseconds pulse of 27 J/cm^2 after the removal of IDM, 470 nm.
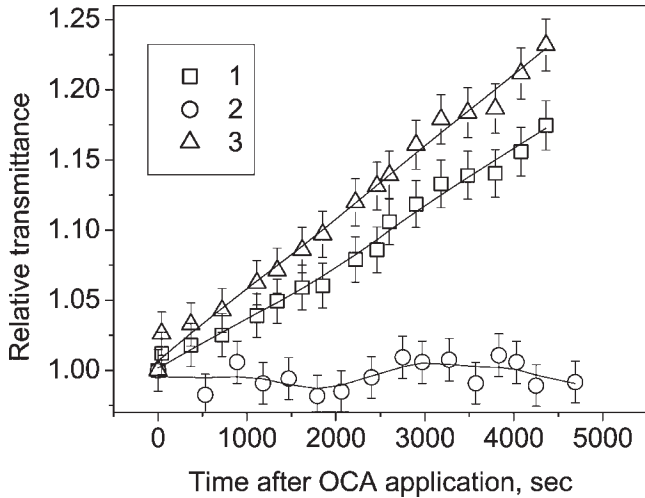
Fig. 14. The effect of LID treatment on the rate of the immersion clearing of pig skin ex vivo by 40%-propylene glycol solution: 1—specimen received one 20-milliseconds pulse of 27 J/cm^2 with IDM and two 20-milliseconds pulses of 27 J/cm^2 after the removal of IDM, 650 nm. 2—control specimen received three 20-milliseconds pulses of 27 J/cm^2 without IDM; 3—specimen received one 20-milliseconds pulse of 27 J/cm^2 with IDM, and two 20-milliseconds pulses of 27 J/cm^2 after the removal of IDM, 470 nm.

spectrum for glycerol reveals, on the whole, a greater slope which may be indicative of higher extent of index matching between the scattering centers and base material for this OCA.
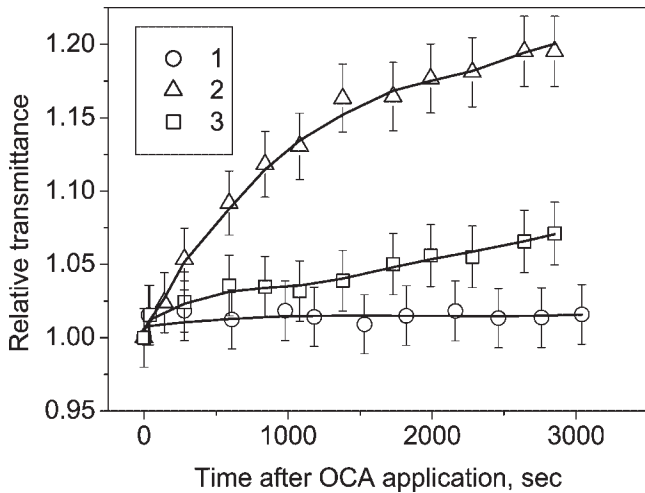

Fig. 15. The effect of LID treatment on the rate of the immersion clearing of pig skin ex vivo by 60%-glycerol solution: 1—control specimen received three 20-milliseconds pulses of 27 J/cm^2 without IDM; 2—specimen received one 20-milliseconds pulse of 27 J/cm^2 with IDM and two 20-milliseconds pulses of 27 J/cm^2 after the removal of IDM, 470 nm; 3—specimen received one 20-milliseconds pulse of 27 J/cm^2 with IDM and two 20-milliseconds pulses of 27 J/cm^2 after the removal of IDM, 650 nm.
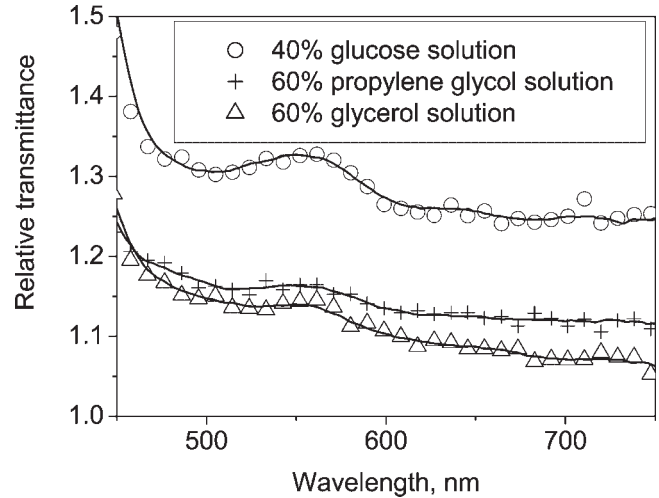

Fig. 16. "Relative transmittance" spectra of LID-treated pig skin with different OCAs applied. Elapsed time 60 minutes.

In all presented spectra, the local maximum in the region of α and β hemoglobin absorption bands and the steep rise of the curve towards the spectral location of Soret band are pronounced that points to the role of absorption in the efficiency of optical clearing. As the human skin is a medium with strong scattering the transmitted photons are substantially multiply scattered and have long pathways within the tissue. Under the process of optical immersion clearing the decrease in scattering causes two effects leading to the increase of transmittance: (i) decrease of beam remittance out of the direction of propagation; (ii) fall in light absorption by the natural skin chromophores for the decrease of optical path within the sample, the latter being pronounced only at the wavelengths of absorption bands.
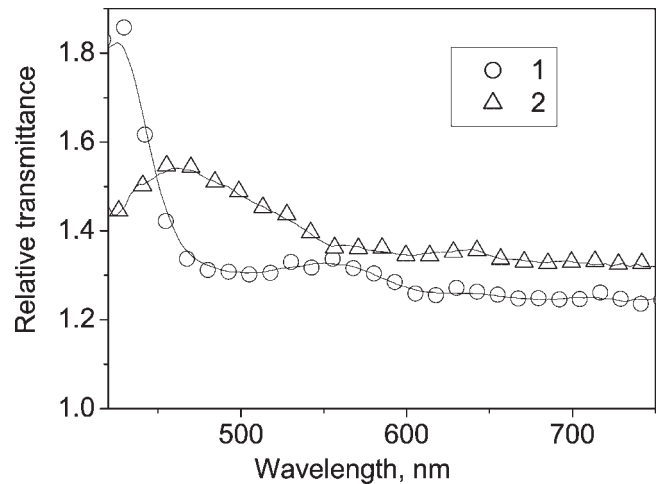

Fig. 17. "Relative transmittance" spectra of LID-treated pig skin obtained under different irradiation geometry: 1—illumination with a wide flat beam; 2—a quasi-collimated narrow-beam illumination. OCA: 40%-glucose solution in water. Elapsed time: 60 minutes.
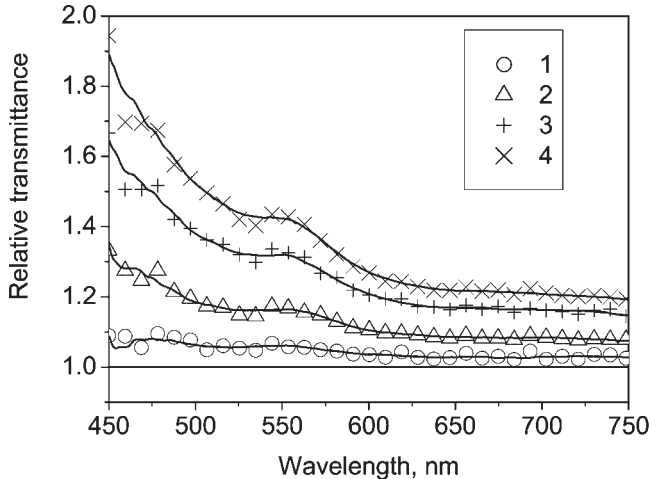
Fig. 18. The temporal evolution of "relative transmittance" spectra of LID-treated human skin with 40%-glucose solution topically applied. Elapsed time is: 1–15 minutes; 2–30 minutes; 3–45 minutes; 4–1 hour.
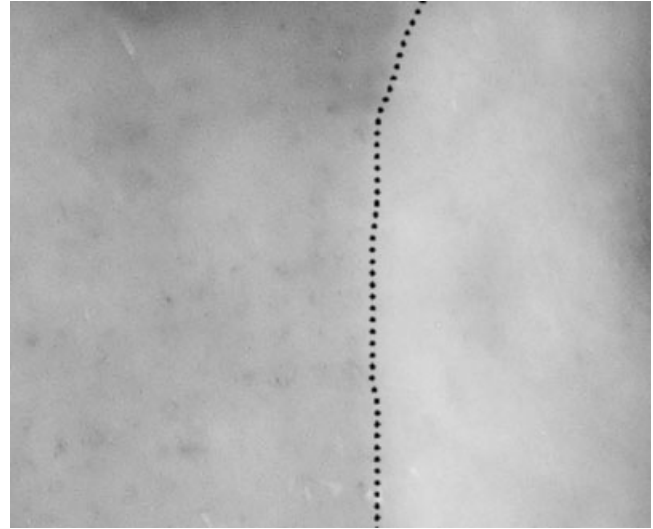

Fig. 19. Reflectance image of the human skin ex vivo. OCA: 40%-glucose solution. The left part (from the border dotted line) of the specimen had received one 20-milliseconds pulse of 27 J/cm^2 with IDM and five 20-milliseconds pulse of 27 J/cm^2 after the removal of IDM. Elapsed time: 60 minutes. Heating by hair-drier ($51 \pm 2°$C on the skin surface) and OCA application was provided for the whole sample.

The effect of hemoglobin absorption on the shape of spectral curves of relative transmittance radically depends on the geometry of measurements. In Figure 17, two spectra that correspond to illumination of the skin sample by a wide flat beam (1) or a narrow quasi-collimated beam (2) at identical detector aperture are shown. While under the illumination by the wide beam the more effective clearing (the increase of transmitted intensity) is attained within the absorption bands, with the narrow beam the higher transmittance is observed over the intervals of minimum absorption. In the case of the narrow beam illumination the fraction of the photons with shorter paths in the detected intensity should be higher (for the absence of the sites of light incidence far out of the source-detector axis) and the decrease in scattering would affect the light absorption within the tissue to much less extent.

It is interestingly that under the conditions of wide beam illumination, the "positive" contribution of hemoglobin absorption into the clearing rises as the process proceeds; this is illustrated by a set of relative transmittance spectra of human skin obtained at different elapsed times (see Fig. 18).

Figure 19 illustrates how human skin backreflectance is changed under OCA action. The left part of the sample was treated by light with IDM and looks darker (lower backscattering) than the left part of the sample, which was not treated by light.

Diffusion Processes and Optical Clearing Mechanisms

Human skin consists of the following three principal layers: SC, viable epidermis, and dermis. SC is a lipid-protein biphasic structure, having a thickness of only 10–20 μm on most surfaces of the human body. Due to cell membrane keratinization, tightly packing of cells and lipid bridges between them, SC is a dense medium with a pour

penetration to foreign molecules [19]. The excellent diffusional resistance of the SC makes the transdermal delivery of immersion agents and water lost by skin difficult. To understand how to control the transport and barrier functions of the skin, it is important to have knowledge of the water distribution within the different layers [19]. The SC receives water from within the body but also water may be taken up from the environment. In vivo the diffusion of water across the SC is a passive process that can be modified at application of osmotic OCAs. The outside SC layer is certainly drier than the innermost cornified layer adjacent moist granular layer. Thus, there exists a concentration gradient causing transepidermal water loss, which can be increased at LID combined with an osmotic OCA application.

Low permeation of the normal skin is determined by the SC, however, viable epidermis and dermis, in spite of their much better permeability than the SC, may significantly delay the OCA diffusion inside the body because of their higher thickness. No significant difference was found for the diffusion across epidermis and SC [33]. The diffusion coefficient D of the flow of water through tissue corresponds to viscose flow through a very fine porous medium. As has been determined in strongly hydrated SC, D is about four orders of magnitude less than self-diffusion coefficient in water [33]. The diffusivity (D) of water in SC increases from $\sim 3 \times 10^{-10}$ to 10^{-9} cm^2/seconds as surrounding relative humidity increases from 46% to 81%. The average water content of the SC in normal state is in the range from 15% to 30% (by weight) as measured from outmost to innermost layers. The normal hydration of the viable cell layers of the

epidermis is not significantly different from that of the dermis, which is of 70% by weight [19,34].

Dermis is the next thicker layer of the skin, which is mostly fibrous tissue, thus can be easily impregnated by exogenous or endogenous liquids (OCAs). Subcutaneous tissue contains a big portion of fat cellular layer, which is much less penetrative for diffusing molecules than dermis. Such specific structure of skin defines the methodology of its effective optical clearing, which is related to the matching of refractive indices of scatterers (keratinocytes components in epidermis, collagen, and elastin fibers in dermis) and ground matter [8,10,15].

Let us estimate the diffusion time (see Appendix) for different skin layers. Supposing that SC thickness is equal to $d = 10$–20 μm and that for small molecules, such as glycerol, propylene glycol, diffusion coefficient is close to water, that is, $D_a = 3 \times 10^{-10}$ cm^2/seconds, then from Equation (6) it follows that in dependence of SC thickness τ_a can be ranged from ~0.9 to ~3.6 hours. For living epidermis of thickness 100 μm and OCA diffusivity of $D_a = 3 \times 10^{-8}$ cm^2/seconds, approximately, the diffusion time τ_a~0.9 hour can be provided. Two orders higher diffusivity of the living epidermis in comparison with the SC is due to a more permeation ability of epidermal cell membrane, which is similar to permeability of membranes of other epithelial cells. For a 1-mm dermis thickness and typical diffusivity of fibrous tissue, $D_a = 3 \times 10^{-6}$ cm^2/seconds [10], τ_a can be estimated as ~0.9 hour. In accordance with these estimations, 2.7–5.4 hours is needed for OCA diffusion through a skin layer. Evidently, in dependence on tissue condition and place on the body, this time can be different. Approximately equal contributions to the time delay of OCA permeation is provided by all three skin major layers with a 10-μm SC. For a thicker, 20-μm SC, resistance of SC dominates, thus various vehicles and methods for reversible disruption of SC protective function should be used to provide reasonable time for OCA diffusion.

One of the possible mechanisms of more effective optical clearing of skin by an osmotic OCA at LID application is connected with more effective dehydration of skin due to local disruption of the SC. A special experiment with Yucatan pig skin samples at LID (two 20-milliseconds pulses of 30 J/cm^2 each with IDM and two 20-milliseconds pulses of 30 J/cm^2 each after IDM removal) done for sample non-treated by OCA and treated with 40%-glucose showed that sample area strictly corresponding to LID-area was dehydrated more effectively than surrounding area free of LID. The dehydrated area was clearly seen as more translucent area with a less thickness. Tissue shrinkage was of 20–25% in thickness of the sample with LID kept at room temperature for 2 hours and up to 40% in thickness of the sample treated with 40%-glucose and kept in oven at temperature 51°C during 2 hours. Thus, more effective skin dehydration is expected at LID due to SC partial ablation. Besides, the local heating of the living epidermis under the SC may enhance skin permeability due to induced phase transition of epidermal intercellular lipids from gel phase to liquid crystalline phase [37,38].

The SC is functioning not only as a barrier against OCA penetration into skin, but also as a reservoir for topically applied substances [19,39]. Skin appendages, in particular sebaceous glands, also serve as a reservoir for clearing agents [40,41].

CONCLUSION

Enhancement of skin permeability using a lattice of islets of limited thermal damage on SC is a promising technique for accelerating delivery of OCA, as well as drug and other compounds, to their target areas within the skin.

Island damages in SC of human and animal skin specimen ex vivo have been produced with the aid of pulse phototreatment with IDM. Island damage method could be used successfully for the creation of small holes in SC in in vivo conditions and consequently for the enhancement of optical clearing of treated skin area using different compositions of biocompatible agents.

The concept of the lattices of optical islets can be used as a safe yet effective treatment modality in dermatology, dentistry, ophthalmology, and other biomedical applications where the target of treatment is sufficiently superficial. Substantial research still needs to be done for better understanding and optimizing this new type of phototreatment.

APPENDIX

Evidently, for effective optical clearing enhanced diffusivity of an agent through skin is important. Basing on Fick's law, which limits the flux of matter J (mol/seconds/cm^2) to gradient of its concentration [20]:

$$J = -D_a \frac{dC}{dx} \qquad (2)$$

the expression for stationary transport of matter through a thin tissue layer of thickness d can be written in a form

$$J = P_a(C_1 - C_2) \qquad (3)$$

where $P_a = D_a/d$ is the coefficient of permeability, and C_1 and C_2 are the concentrations of molecules in two spaces separated by a tissue layer.

Using Equations (2) and (3), it is possible to find the variation in concentration of molecules inside a closed space with a volume V surrounded by a permeable membrane with an area S using the following equation [20]

$$\frac{dC_2}{dt} = \frac{P_a S}{V}(C_1 - C_2) \qquad (4)$$

For a large external volume when C_1 can be considered as a constant, Equation (3) has an approximate exponential solution for the volume-averaged concentration of an agent $C_a(t)$, which has a view [5,35]

$$C_a(t) = \frac{1}{2}\int_0^d C_a(x,t)dx \cong C_{a0}\left[1 - \exp\left(-\frac{t}{\tau}\right)\right] \qquad (5)$$

When agent is administrated through only one sample surface, which is characteristic to in vivo agent application,

the diffusion time is defined as [12]:

$$\tau = \frac{d^2}{D_a} \qquad (6)$$

The diffusion coefficient D_a is a function of the skin condition and size and form of a molecule. For the spherical molecules diffusing in biological membrane the following equation is valid [20]:

$$D_a = \text{const} \times M^{-S} \qquad (7)$$

In general, parameter S for diffusion through a biological membrane is about 3.5. For example changing of molecule weight M from 45 to 122 at diffusion through the plasmatic membrane changes the diffusion coefficient from 1.4×10^{-8} to 2.0×10^{-10} cm^2/seconds (70-folds) [20]. The rate of diffusion of molecules with molecular weight of 119 in the SC of volunteers is in the range from $\sim 10^{-10}$ to 3.5×10^{-10} cm^2/seconds [36], that is approximately equal to water diffusion rate in SC.

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