This paper presents results of in vivo and in vitro study of rat skin clearing under action of osmotic active liquid. 40%-glucose solution has been used as an osmotic agent. The degree and time of clearing of different skin samples have been studied in vitro. In vivo administration of the clearing agent has been made by hypodermic injection to exclude the protective action of stratum corneum barrier and influence of the adipose layer, which also reduces rate of the agent diffusion into the tissue. The significant increase of transmittance of the skin samples in vitro and decrease of reflectance of skin in vivo in about an hour after glucose administration were demonstrated. The diffusion coefficients of 40%-glucose solution in the rat skin in vitro have been estimated.

1 Introduction

The control of tissue optical properties is important for the development of methods of optical tomography, photodynamic therapy and selective photodamage of tissue components. The main difficulty in clinical application of the optical diagnostic and therapy techniques for monitoring of human body and the laser treatment is caused by strong light scattering of the majority of tissues. In the visible and NIR spectral ranges the scattering coefficient of a tissue is a few orders higher than the absorption coefficient.

The decrease of the scattering coefficient of skin can be achieved by matching of refractive indices of scatterers (keratinocyte components in epidermis, collagen and elastin fibers in dermis) and ground matter. The equalizing of the refractive indices of the scatterers and surrounding medium occurs at administration of immersion liquid with refractive index greater than one of interstitial or intercellular substance of tissue. Interaction between the tissue and immersion liquid is accompanied by the change of tissue thickness, sizes and packing density of scatterers, but usually the refractive index matching effect prevails over other processes and tissue clearing is observed. For example, optical transmittance of skin increases in a few folds and that of sclera increases in a few dozens folds. The total clearing of sclera occurs, as a rule, in 8-10 minutes, and that of skin occurs in about an hour. As the secondary effects, drying of tissue and cells, their swelling or shrinkage should be taken into account.

The temporal increase of the skin transparency can improve the optical penetration depth, i.e. image contrast and resolution in confocal microscopy, OCT and other optical diagnostic techniques and could be useful in laser therapy and surgery as well. Application of the appropriate chemical agents can effectively change the scattering properties of living tissues. Experimental studies on optical clearing of normal and pathological skin and its components (epidermis and dermis) and the management of reflectance and transmittance spectra using water, glycerol, glycerol-water solutions, glucose, sunscreen creams, cosmetic lotions, gels and pharmaceutical products have been presented in Refs. 16-19.

However, methods of effective application of immersion agents to skin should be designed, and dynamic characteristics of clearing should be studied. The knowledge of the dynamic characteristics of clearing is important for the carrying out of the protocols for both diagnostic and treatment of malignant growths hidden under skin surface.

This paper presents results of in vivo and in vitro study of rat skin clearing under action of 40%-glucose solution. The degree and the clearing time of skin samples with and without the adipose layer have been studied. In general, dynamic behavior of in vivo and in vitro clearing has to be similar, but there are expected the essential quantitative distinctions in the clearing degree and time concerned with method of tissue impregnation and reaction of the living tissue on the clearing agent.
2 Materials and Methods

2.1 Preparation of tissue samples and rat specimen

Rat skin samples were obtained by autopsy within an hour post mortem. Hair was removed using tweezers. The skin tissue was cut into pieces (thickness from 0.58 to 0.76 mm) with the area about 1 cm². The samples was fixed on the plastic frame and placed into the glass cuvette filled up by the immersion liquid (Fig. 1).

In vivo measurements were done with white rat. Age of the rat was about 9 months and its weight was about 200 g. Rat was anaesthetized by an intraperitoneal injection of 1%-natrium ethaminal solution (40 mg/kg of animal weigh) prior to procedure. Removing hair was done before experiments. The 40%-glucose solution (0.1 ml) was injected under skin dermis in the area of thigh. The measurement of dynamics of the optical clearing was started in 60 sec after the injection.

Refractive index of the 40%-glucose solution used as an immersion liquid was measured on Abbe refractometer (1.39 at wavelength 589 nm), pH was measured as 3.4 by standard method.

2.2 Experimental setup

Measurements of reflectance spectra were performed using a commercially available spectrometer LESA-5 (BioSpec, Russia). Scheme of the experimental setup is shown in Fig. 1.

To provide in vitro measurements of light transmission the framed skin sample (4) was put into cuvette (3) filled up by glucose solution (5) and placed between two optical fibers (400 µm core diameter). We used the 0.5 mm diaphragm (6) placed 20 mm apart from the tip of the receiving fiber (7) providing collimated transmittance measurements. Neutral filter (2) was used to attenuate the incident radiation. Measurements of the spectra were performed every 60 - 120 sec during time-intervals from 200 to 400 min.

In vivo reflectance measurements were performed using a fiber optical probe with a system of optical fibers. The fibers (1) were enclosed in cone-shaped aluminum jacket (8) to provide a fixed distance
between the fibers and skin surface. Light from a stabilized light source (xenon arc lamp) was delivered to the tissue by means of the fiber fixed normally to the skin surface. The receiving fiber was displaced at angle of 20 degrees to the surface in such way as the irradiated area had 6-mm diameter and the area of light collection had 10-mm diameter.

Reflectance spectra of skin were measured against BaSO₄ plate as a reference. To register the reflectance spectra of the rat skin in vivo the fiber optical probe was placed on the surface of the skin. The measurements were performed every 60 sec during 105 min. All experiments were performed at room temperature.

2.3 Estimation of 40%-glucose solution diffusion coefficient

To estimate of 40%-glucose solution diffusion coefficient, we used the experimental data for the collimated transmittance, which are presented in Figure 2. Transport of glucose solution within the tissue can be described in the framework of the matter diffusion model. The diffusion equation for the local variation of glucose concentration within a skin layer can be presented in the form

$$\frac{\partial C}{\partial t} = D \frac{\partial^2 C}{\partial x^2},$$

where C is the glucose concentration, D is the diffusion coefficient \( [D] = \text{cm}^2/\text{sec} \), and x is the spatial coordinate \([x] = \text{cm}\).

Solution of the diffusion equation for a plane slab with a thickness \(d\), at the moment \(t\) (the initial concentration of this substance within the slab is equal to 0, i.e., \(t = 0\); \(0 \leq x \leq d\); \(c = 0\)) has the form

$$C = C_0 \left\{ \frac{4}{\pi} \left[ \exp\left(-t/\tau\right)\sin(\pi x/d) + \frac{1}{3} \exp\left(-9t/\tau\right)\sin(3\pi x/d) + \frac{1}{5} \exp\left(-25t/\tau\right)\sin(5\pi x/d) + \ldots \right] \right\},$$

where \(\tau = \frac{d^2}{\pi^2 D}\) is the diffusion constant, \(C_0\) is the glucose concentration in external volume, \(d\) is the thickness of the skin sample. The first approximation of this equation can be presented as

$$C = C_0 \left\{ 1 - \exp\left(-\frac{t}{\tau}\right) \right\},$$

that is very close to the equation describing diffusion through a partially permeable membrane.

We assumed that the following approximations are valid for description of glucose transport:

a) The diffusion coefficient is constant over the entire skin volume.

b) Volume fraction of the glucose solution in the interstitial fluid is defined as

$$C_{\text{gl}}(t) = \frac{V_{\text{gl}}(t)}{V_{\text{gl}}(t) + V_{\text{w}}(t)} = C_{\text{w}} \left\{ 1 - \exp\left(-\frac{t}{\tau_{\text{w}}} \right) \right\},$$

where \(V_{\text{gl}}(t)\) is the time-dependent volume of glucose solution in the tissue interstitial space; \(V_{\text{w}}(t)\) is the time-dependent volume of water and proteins that constitute the interstitial matter; \(C_{\text{w}}\) is the concentration of glucose solution in the external volume (expressed in volume fractions); \(\tau_{\text{w}} = d(t)^2/D_{\text{w}}(t)\) is the diffusion constant; \(D_{\text{w}}(t)\) is the time-dependent glucose diffusion coefficient; \(d(t)\) is the time-dependent thickness of the skin sample. The volume fraction of water and proteins: \(C_{\text{w}}(t) = 1 - C_{\text{gl}}(t)\).

c) The time dependence of the skin tissue thickness can be described by the following phenomenological expression:
\[ d(t) = d(t=0) + A \left( 1 - \exp \left( \frac{t}{\tau_{sw}} \right) \right), \]

where \( A \) and \( \tau_{sw} \) are some phenomenological constants for describing the swelling process caused by glucose action.

a) The refractive index of the interstitial fluid is defined as
\[ n_{i}(t) = (1 - C_{s}(t)) \cdot n_{\text{base}} + C_{s}(t) \cdot n_{\text{gl}}, \]
where \( n_{\text{base}} = 1.36^{16} \) and \( n_{\text{gl}} = 1.39. \)

b) The scattering coefficient dependence on refractive index of interstitial fluid is defined by the following equation:
\[ \mu_{s}(t) = N \cdot \sigma_{r}(t), \]
where \( N \) is the number of particles in a volume unit and \( \sigma_{r}(t) \) is the time-dependent cross-section of scattering which is determined by Mie theory. To define \( \sigma_{r}(t) \) it is need to know both a mean size of the scatterers and a value of time-dependent relative refractive index \( m(t) = n_{r}(t)/n_{i}(t). \) The mean size of the scatterers have been estimated in Ref. 26 as 0.5 \( \mu \)m. Refractive indices of the collagen particles and the interstitial fluid were measured as 1.47 and 1.36,\(^{16}\) respectively. We supposed that the refractive index of the scatterers and their size did not change during the clearing process.

c) \( N \) can be estimated as
\[ N = \frac{\mu_{s}}{\sigma_{r}} \left( \frac{\text{experimental value for } \lambda=589 \text{ nm at initial moment}}{\text{calculated value for } \lambda=589 \text{ nm at initial moment}} \right). \]

d) The collimated transmittance of the skin sample impregnated by the glucose solution can be defined as
\[ T_{c}(t) = (1 - R_{s}) \cdot \exp \left( - (\mu_{a} + \mu_{s}(t)) \cdot d(t) \right), \]
where \( R_{s} \) is the specular reflectance, \( \mu_{a} \) and \( \mu_{s} \) are the absorption and scattering coefficients, respectively. Values of the absorption coefficients of skin were presented in Ref. 19.

This set of relations describing the glucose concentration in dependence on time represents the direct problem. The reconstruction of the diffusion coefficient of the glucose solution into the skin was carried out on the basis of measured time evolution of the collimated transmittance. The solution of the inverse problem were done by minimization of a target function: \( F(t) = (T_{c}(t) - T_{c}^{*}(t))^{2}, \) where \( T_{c}(t) \) and \( T_{c}^{*}(t) \) are the theoretical and experimental values of the time-dependent collimated transmittance, respectively. To minimize the target function the Simplex method described in details in Ref. 27 was used.

3 Results and Discussion

3.1 In vitro study of the optical clearing of rat skin

\textit{In vitro} experiments have demonstrated the increase in light transmission through the skin under action of the 40%-glucose solution. Figure 2 shows the time-dependent transmittance of the rat skin samples at the wavelength 700 nm.
Fig 2. The time-dependent collimated transmittance of both rat skin samples: with (circles) and without (squares) adipose layer measured at the wavelength 700 nm after immersion into the 40%-glucose solution. Symbols correspond to experimental data; solid line corresponds to fitting of the experimental data. Thickness of the samples was 0.73 and 0.57 mm, respectively.

It is well seen that at the early stages of skin impregnation by the immersion liquid it is poorly transparent for visible light. The influence of the 40%-glucose solution leads to the increase of collimated transmittance of the samples (i.e. to the clearing of the tissue). The optical properties of skin in general are defined by dermis because of relatively big thickness of dermis (95% of the human skin)\textsuperscript{16}. Therefore, both degree and speed of the clearing of skin depend on manner of administration of the immersion liquid into the dermis. Experimental results have shown that glucose solution penetrates easily into the skin sample from its internal side (i.e. from the dermis), since its penetration through the epidermis is limited by the protective nature of the stratum corneum and epidermis (cell structure). The presence of a layer of adipose (subcutaneous fat) on the sample influenced on the time of the sample clearing.

We researched both types of samples with and without adipose layer. The thickness of the adipose layer changed in different skin samples from different animals from about 0.1 to 0.5 mm. The thickness of the skin without the adipose layer varied in wide range from about 0.3 to 0.7 mm. However, for all samples, from which the adipose layer was removed, the total clearing of tissue took place significantly earlier than for the samples with the adipose layer. Typical dependencies of the changes of the collimated transmittance of the samples from the both groups on the clearing time are presented in Figure 2. The thickness of the both samples with and without the adipose layer was 0.73 and 0.57 mm, respectively. From this figure it is well seen that diffusion of glucose solution through the adipose tissue took place very slowly (up to 3 hours). The mean thickness of the adipose layer was about 0.13 mm. Dehydration and corresponding decrease of the thickness of the adipose layer during the clearing were observed. Collimated transmittance at 700 nm increased up to 14 folds in 320 min for the sample with the adipose layer, and up to 35 folds in 80 min for the sample without the adipose layer.

The diffusion coefficients for the glucose transport were estimated as $D = 1.9 \cdot 10^{-9} \pm 1.02 \cdot 10^{-7} \text{cm}^2/\text{sec}$ and $D = 3.25 \cdot 10^{-9} \pm 7.36 \cdot 10^{-7} \text{cm}^2/\text{sec}$ for the samples with and without the adipose layer, respectively.
Thus, for the effective clearing of the skin *in vivo* at the application of the immersion agent we have to exclude the influence of both epidermis and adipose layer, i.e. glucose injection should be precisely hypodermic.

### 3.2 In vivo study of the optical clearing of rat skin

Figures 3 and 4 show dynamics of reflectance spectra and time-dependent reflectance at different wavelengths of the rat skin after hypodermic injection of 40%-glucose solution. In the spectra the main absorption bands of blood are well seen (Fig. 3). The decrease of the spectra corresponds to diffusion of the glucose solution from the injection place to the upper skin layers.

Correspondingly with Figure 4, the tissue clearing took place during about an hour. At that time, the value of the reflectance decreased in about two folds at the wavelength 700 nm.

![In vivo reflectance spectra of rat skin measured at different time intervals after injection of 40%-glucose solution. Symbols correspond to experimental data: squares – before injection, circles – 15 min, up triangles – 30 min, down triangles – 45 min, and diamonds – 105 min.](image)
Control of skin optical properties: in vivo and in vitro study

Fig 4. *In vivo* time-dependent reflectance of rat skin measured at different wavelengths after injection of 40%-glucose solution. Symbols correspond to experimental data: squares – 420 nm, circles – 500 nm, diamonds – 700 nm.

![Graph showing time-dependent reflectance](image)

Fig 5. The sensitivity to hemoglobin absorption (calculated as hemoglobin spectral peaks (dips) contrast) via time-interval of the clearing. $R_{\lambda_1}$, $R_{\lambda_2}$ are maximal and minimal values of the skin reflectance at the wavelengths $\lambda_1 \approx 610$ nm and $\lambda_2 \approx 575$ nm, respectively. Symbols correspond to experimental data; solid line correspond to fitting of the experimental data.

![Graph showing sensitivity to hemoglobin absorption](image)
On the base of *in vitro* experiments it can be concluded that the matching of refractive indices of collagen fibers of dermis \((n = 1.46)\) and interstitial liquid \((n = 1.36)\) makes the main contribution to the tissue clearing in the first phase (in the first hour after injection)\(^{16,23}\).

The reflectance of the skin increased due to the glucose going out from the area of observation. In this stage the diffusion of the clearing agent along the skin surface and in the depth took place. The reversion of the reflectance passed very slowly (in a few hours) and is not shown in figure (see Fig. 4).

The decrease of scattering allows one to see (image) absorption sites more precisely. In this experiments the absorption site is blood perfusion within tissue. The sensitivity of this method of reflectance spectrometry to the absorption of blood in vessels hidden in the skin thickness was estimated. This sensitivity was calculated as hemoglobin peaks (dips) contrast: 
\[
\left( R_{\lambda_1} - R_{\lambda_2} \right) / \left( R_{\lambda_1} + R_{\lambda_2} \right),
\]
where \(R_{\lambda_1}, R_{\lambda_2}\) are maximal and minimal values of the skin reflectance at the wavelengths \(\lambda_1 \approx 610\) nm and \(\lambda_2 \approx 575\) nm, respectively. Dependence of the sensitivity of this method to the blood absorption on the clearing time is shown in Fig. 5. It is seen that the contrast of the hemoglobin peaks increased during the first hour in corresponding to dynamics of the reflectance change. Thus, due to the dermis immersion it is possible to increase the imaging contrast of vessels, tumors and other blood-perfused abnormalities.

### 4 Conclusion

The results of this paper show that administration of osmolytes to a fibrous tissue allows for effective control of its optical characteristics. The scattering of skin are substantially decreased by the refractive indices matching of the scatterers and the interstitial substance. The diffusion coefficients of 40%-glucose solution in the rat skin *in vitro* were estimated as 
\[
D = 1.9 \cdot 10^{-6} \pm 1.02 \cdot 10^{-7} \text{ cm}^2/\text{sec} \quad \text{and} \quad D = 3.25 \cdot 10^{-6} \pm 7.36 \cdot 10^{-7} \text{ cm}^2/\text{sec}
\]
for the samples with and without the adipose layer, respectively. The most effective technique for immersion agent administration is hypodermic injection. The return to the initial (turbid) state takes place very slowly (in a few hours) that allows one to carry out diagnostics and treatment of malignant growths hidden under the skin surface. The highest contrast of blood content abnormalities is expected in an hour after glucose injection.

### Acknowledgement

The research described in this publication was made possible in part by Award "Leading Scientific Schools" number 00-15-96667 of the Russian Basic Research Foundation and by Award No. REC-006 of the U.S. Civilian Research & Development Foundation for the Independent States of the Former Soviet Union (CRDF).

### References

Control of skin optical properties: in vivo and in vitro study

16 Tuchin V V, Tissue optics: Light Scattering Methods and Instruments for Medical Diagnosis (V. TT38, SPIE Press, Bellingham), 2000.