НАУЧНЫЙ ОТДЕЛ

БИОФИЗИКА И МЕДИЦИНСКАЯ ФИЗИКА

Известия Саратовского университета. Новая серия. Серия: Физика. 2023. Т. 23, вып. 2. С. 120–127
Izvestiya of Saratov University. Physics, 2023, vol. 23, iss. 2, pp. 120–127
https://fizika.sgu.ru https://doi.org/10.18500/1817-3020-2023-23-2-120-127, EDN: SHGJIM

Article

Ex vivo study of the kinetics of ovarian tissue optical properties under the influence of 40%-glucose

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Abstract. ***Background and Objectives*:** The reproductive system of women is a subject of diverse multidisciplinary research worldwide. These are oncological diseases, infertility of an unspecified nature, ovarian cryopreservation to preserve fertility, improvement of early diagnosis of diseases, etc. Elucidation of the mechanisms of diffusion of tissue water and hyperosmotic agents in luteal phase ovarian tissues controlled by angiogenic growth factors can bring us closer to understanding biophysical processes in general. ***Materials and Methods*.** The work used spectroscopy of diffuse reflection, a free diffusion model and the modified Bouguer–Lambert–Beer law. ***Results*.** The diffusion coefficient of 40% glucose/tissue water into the ovarian tissue of the luteal phase D and the diffusion time τ have been determined, which was $D = (8.6 \pm 1.4) \cdot 10^7$ cm^2/s and $\tau = 50.4 \pm 1.7$ min with a sample thickness of (0.8 ± 0.1) mm. The efficiency of optical clearing of cat ovarian tissues with 40% glucose immersion has been determined. ***Conclusions*.** Studies have shown that 40%-glucose is an effective optical clearing agent for topical use in differentiating normal and pathological ovarian tissues and in clinical applications.

Keywords: ovarian tissues, luteal phase, glucose, total transmittance spectra, diffuse reflectance spectra, diffusion coefficient, optical clearing efficiency

Acknowledgements: The study was financially supported by the Russian Science Foundation under project No. 22-75-00021 dated July 28, 2022.

For citation: Selifonov A. A., Rykhlov A. S., Tuchin V. V. *Ex vivo* study of the kinetics of ovarian tissue optical properties under the influence of 40%-glucose. *Izvestiya of Saratov University. Physics*, 2023, vol. 23, iss. 2, pp. 120–127. https://doi.org/10.18500/1817-3020-2023-23-2-120-127, EDN: SHGJIM

Научная статья
УДК 535.341.08:535.346.1

***Ex vivo* исследование кинетики оптических свойств тканей яичников под действием 40%-глюкозы**

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Аннотация. ***Предыстория и цели.*** Репродуктивная система женщин является предметом разнообразных междисциплинарных исследований во всем мире – это онкологические заболевания, бесплодие неуточненной природы, криоконсервация яичников для сохранения фертильности, совершенствование ранней диагностики заболеваний и др. Выяснение механизмов диффузии тканевой воды и гиперосмотических агентов в тканях лютеиновой фазы яичников, контролируемых ангиогенными факторами роста, может приблизить нас к пониманию биофизических процессов в целом. ***Материалы и методы.*** В работе использовались спектроскопия диффузного отражения, модель свободной диффузии и модифицированный закон Бугера–Ламберта–Бера. ***Результаты.*** Определены коэффициент диффузии 40%-ной глюкозы/тканевая вода в ткань яичника лютеиновой фазы D и время диффузии τ, которое составило $D = (8.6 \pm 1.4) \times \times 10^7$ см^2/с и τ = 50.4 ± 1.7 мин при толщине образца (0.8 ± 0.1) мм. Определена эффективность оптического просветления тканей яичников кошки при иммерсии 40%-ной глюкозы. ***Выводы.*** Исследования показали, что 40%-ная глюкоза является эффективным оптическим просветляющим агентом для местного применения при дифференциации нормальных и патологических тканей яичников и в клинических применениях.

Ключевые слова: ткани яичника, лютеиновая фаза, глюкоза, спектры полного пропускания, спектры диффузного отражения, коэффициент диффузии, эффективность оптического просветления

Благодарности: Исследование выполнено при финансовой поддержке Российского научного фонда по проекту № 22-75-00021 от 28.07.2022 г.

Для цитирования: *Селифонов А. А., Рыхлов А. С., Тучин В. В. Ex vivo* исследование кинетики оптических свойств тканей яичников под действием 40%-глюкозы // Известия Саратовского университета. Новая серия. Серия: Физика. 2023. Т. 23, вып. 2. С. 120–127. https://doi.org/10.18500/1817-3020-2023-23-2-120-127, EDN: SHGJIM

Introduction

Ultrasound is one of the most common methods for diagnosing or monitoring treatment. This method of examining the internal reproductive organs is a safe and painless method and is prescribed even during pregnancy [1]. The procedure takes 15–20 minutes and is based on the principles of echolocation, when an ultrasonic signal passes through soft tissues at different speeds and is reflected by tissue inhomogeneities. It is impossible to differentiate by ultrasound: endometrial polyps; small neoplasms; fibroids; benign ovarian tumors from cancer, therefore more research is often needed. Optical radiation is of considerable interest for introduction into clinical practice since it allows for obtaining images with up to the subcellular resolution [2]. The method of laparoscopy with an optical channel is an indispensable modern method in clinical gynecology. Laparoscopy is used both in diagnostics, where the optical channel plays an important role, and for obtaining a biopsy material directly from pathological areas of organs and in surgical operations with the least trauma [3]. When light interacts with tissues, the nature of its propagation is determined by such phenomena as reflection, scattering and absorption. Most tissues are optically turbid media that interfere with imaging of deeper layers. The method of optical clearing (OC) is used to reduce light scattering in tissues and create their temporary transparency. This phenomenon can be useful to improve the diagnosis or treatment tactics [4–6]. Several studies OC made using various optical clearing agents (OCA) for the treatment of various types of biological tissues to reduce light scattering [7–9].

The OC method is often implemented using hyperosmotic agents: glucose, sorbitol, glycerin, polyethylene glycol, propylene glycol, dimethyl sulfoxide, etc. OC of tissues operates through two main mechanisms: tissue dehydration and alignment of refractive indices of major tissue components and OCAs. Therefore, the determination of the kinetic parameters of fluid flows is an urgent task, both for understanding the mechanisms of OC and for clinical treatment procedures using hyperosmotic agents [6, 10].

Glucose is a good OCA, which is used in various concentrations in the implementation of the OC method [10]. Clarification of the mechanisms of diffusion of tissue water and hyperosmotic agents is of interest not only in improving diagnostic methods, but also in ovarian cryopreservation to preserve fertility in women who are forced to undergo chemotherapy or have anatomical features [11], as well as in the development of new reproductive technologies for the treatment of infertility [12].

In this work, we study the effect of 40%-glucose on the optical properties of the animal ovarian tissue *ex vivo*.

Methodology

The processes occurring in the ovaries are complex and diverse. The whole cycle of changes can be conditionally divided into phases: follicular, ovarian and luteal. The follicular phase is characterized by the maturation and growth of the follicles and the growth of the egg. The ovular phase relates to the release of a mature egg and the rupture of a follicle (Fig. 1). The luteal phase is characterized by the formation of the corpus luteum, a temporary endocrine gland. The corpus luteum functions for only a few (4–7) days and undergoes involution (in the absence of pregnancy). A white body (scar) appears in its place [13]. The blood supply of the mature corpus luteum is the highest of all the organs of the body. Increased blood supply to the ovary containing the corpus luteum is due to angiogenic growth factors, including the development of a large network of capillaries [14].

For histological examination, 5 tissue samples from different cats aged 1–9 years with a diagnosis of “clinically healthy” were used. The halves of each ovary were cut out manually with a scalpel and fixed. The remaining halves of the ovaries were kept frozen until optical measurements were taken. The material for histological examination was prepared no later than 48 hours after oophorectomy and ovariohysterectomy. For tissue fixation, a 10%-buffered formalin was used. To obtain histological scans, an Aperio AT2 digital slide converter (on-screen diagnostic scanner) equipped with a LED light source and calibration tools was used.

The thickness of tissue histological sections was of 2–3 μm. According to the results of histological studies, it was proved that the selected “normal, unchanged” tissues of the samples contain the corpus luteum and correspond to the luteal phase. The thickness of tissue sections (samples) was measured with a micrometer (Union Source CO, LTD, China), measurements were taken at several points of the sample and averaged. The accuracy of each measurement is ± 0.1 mm. The average thickness of sections of ovarian tissue was (0.8 ± 0.1) mm. To measure the total transmittance spectra (TTS) and diffuse reflectance spectra (DRS) of tissue samples in the spectral range of 200–800 nm, a Shimadzu UV-2550 double-beam spectrophotometer (Japan) with an integrating sphere was used (Fig. 2).

All measurements were carried out at room temperature (~25°C) and normal atmospheric pressure. Each sample of the studied tissue was fixed in a special frame with a window of 0.5×0.5 cm in a quartz cuvette so that the tissue sample was pressed against the wall of the cuvette and subjected to optical measurement. Then, 40%-glucose was added between the sample surface and the cuvette wall, after which measurements were performed until the change in the spectral dependences saturated. We used a pharmaceutical preparation of 40%-glucose aqua solution for injections (Armavir Biofactory,

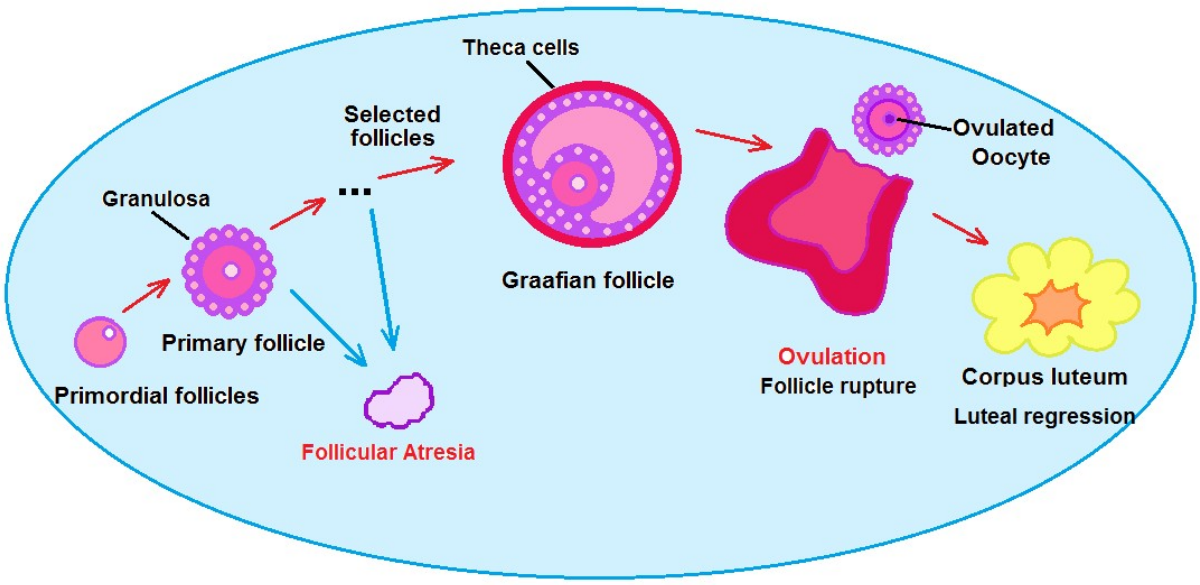


Fig. 1. Schematic representation of the ovarian cycle done using Ref. [13] (color online)

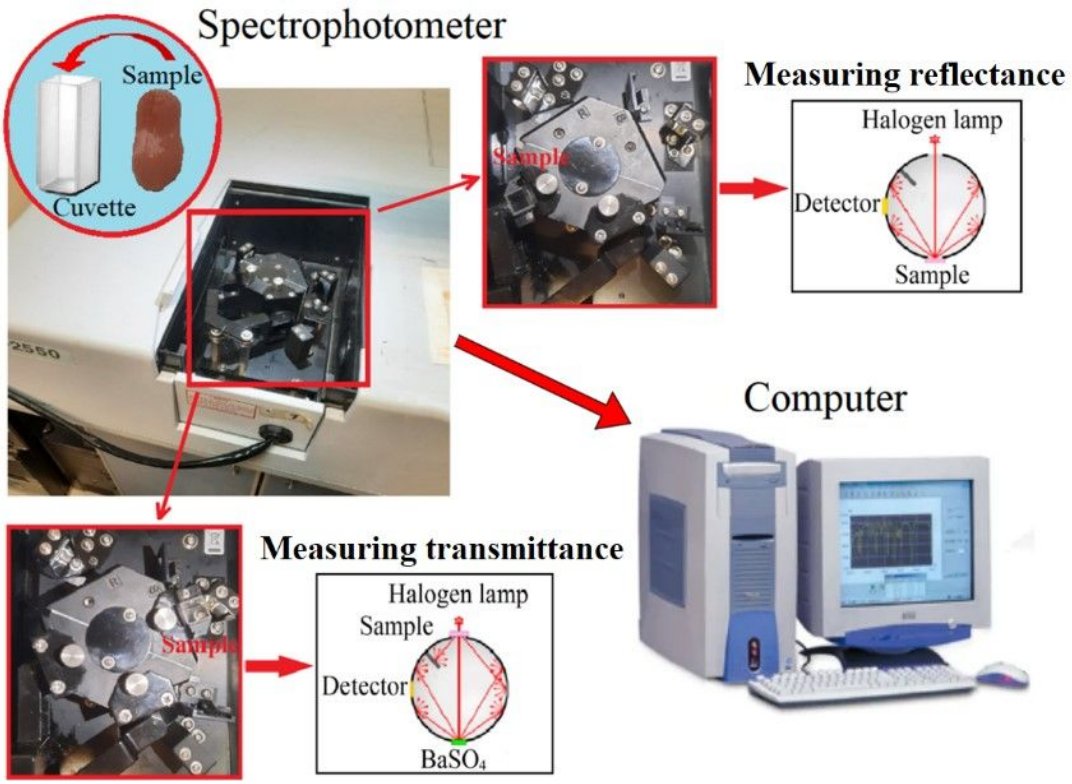


Fig. 2. Scheme of the experimental setup for measuring diffuse reflectance and total transmittance spectra of cat ovarian tissue samples (color online)

Russia). The dehydration of tissue is associated with the outflow of bound intercellular water at the beginning of the interaction with the agent, and the refractive index matching is associated with the agent to flow into the tissue, which generally takes longer time [5] (Fig. 3).

The determination of the tissue diffusion coefficient of glucose/interstitial water is based on the measurement of DRS kinetics. The model of free diffusion was used for calculations [10]. Geometrically, a tissue sample can be represented as a plane-parallel plate of a finite thickness. Using Fick's second law and performing transformations based on the modified Bouguer–Lambert–Beer law [15], we obtain an expression for the difference between the effective optical density at the current time $A(t, \lambda)$ and at the initial time $A(t = 0, \lambda)$:

$$\Delta A(t,\lambda) = A(t,\lambda) - A(t=0,\lambda) = \\ = \Delta\mu_{\text{eff}}(t,\lambda) L \sim C_0 \left\{1 \exp\left(-\frac{t}{\tau}\right)\right\} L, \quad (1)$$

$$I = I_0 \exp[\mu_{\text{eff}} L], \mu_{\text{eff}}(t,\lambda) = \\ = \sqrt{3\mu_a(\mu_a + \mu_s')} \to \Delta\mu_{\text{eff}}(t,\lambda),$$

where the effective optical density is determined from the measurements of DRS:

$$A = -\log R_d; \quad (2)$$

$$\tau = \frac{4l^2}{\pi^2 D}; \quad (3)$$

t is the time in seconds during which the diffusion occurs, λ is the wavelength in nm, $\Delta\mu_{\text{eff}}(t,\lambda)$ is the difference between the effective coefficient of attenuation of light in tissue $\mu_{\text{eff}}(t,\lambda)$ at the current time and at the initial time, 1/cm; L is the average path-length of photons, which in the backscattering mode is $L \cong 2l_d$, $(l_d)^1 = \mu_{\text{eff}}$; $\mu_s' = \mu_s(1-g)$, 1/cm; g is the scattering anisotropy factor (varies from 0 to 1, for many tissues, $g \cong 0.93$) [2]; and for transmission mode $L \cong l$, l is the thickness of the sample, cm; D is the diffusion coefficient of the glucose/interstitial water molecules, cm^2/s; C_0 is the initial concentration of the glucose, mol/l. The recorded DRS ($R(\lambda)$, %) were converted using the standard Kubelka–Munk algorithm to $A(\lambda)$ extinction spectra (Shimadzu UV-2550 spectrophotometer software). To record the final DRS and TTS after the completion of time-dependent spectra measurement during glucose immersion, tissue samples were placed in clean quartz cuvettes and the spectra were recorded

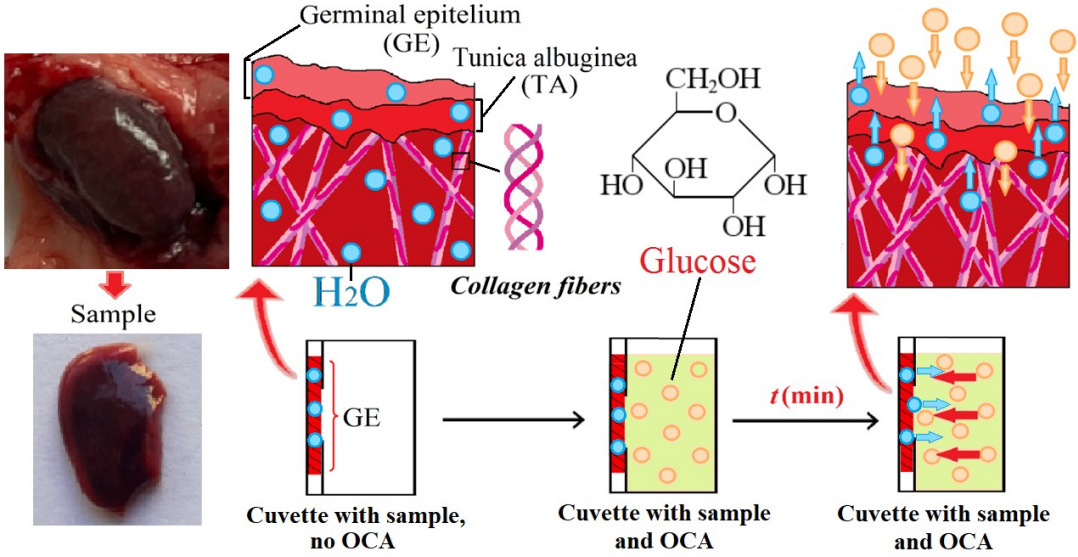


Fig. 3. Diagram showing the interaction of glucose (OCA) with ovarian tissue (color online)

without glucose to observe changes in the spectrum of the sample only, since the spectrum of glucose has absorption maxima in the UV region.

The efficiency of OC (Q) is evaluated [5] as follows:

$$Q(\%) = \{[T(t) - T(t=0)]/T(t=0)\}100\%, \quad (4)$$

where $T(t=0)$ is the tissue sample transmittance at the initial time, $T(t)$ is the current time transmittance.

To determine the confidence interval of the measured values, it is necessary to find the standard deviation (SD) of random measurement errors:

$$SD = \sqrt{\sum_{i=1}^{n} \frac{(x_i - \bar{x})^2}{(n-1)}}, \quad (5)$$

where x_i is the i value of the sample; $\bar{x}$ is the arithmetic mean of the sample; n is the number of experiments.

The bars on the graphs represent the boundaries of the confidence interval (σ) found as:

$$\sigma = (t_s SD)/(\sqrt{n}), \quad (6)$$

where t_s is the Student's coefficient (table value); SD is the standard deviation, $n = 5$, $p = 95\%$.

To determine if there is a significant difference in the mean values of the diffuse reflectance and the total transmittance spectra of the samples before and after exposure to 40%-glucose, the means were evaluated by using the t-test for paired sets of samples from the standard package of data analysis tools of Microsoft Excel. To do this, for each wavelength in the range 200–800 nm the experimental data for samples before (n=5) and after (n=5) OC were compared. The critical test value was found from the table of values for the t-distribution, which corresponds to the critical two-tailed test t_{cd} with a significance level ($\alpha = 0.05$). Student's criterion is determined by the formula:

$$t_s = \frac{\bar{x_1} - \bar{x_2}}{\sqrt{\frac{SD_1^2}{n_1} + \frac{SD_2^2}{n_2}}}, \quad (7)$$

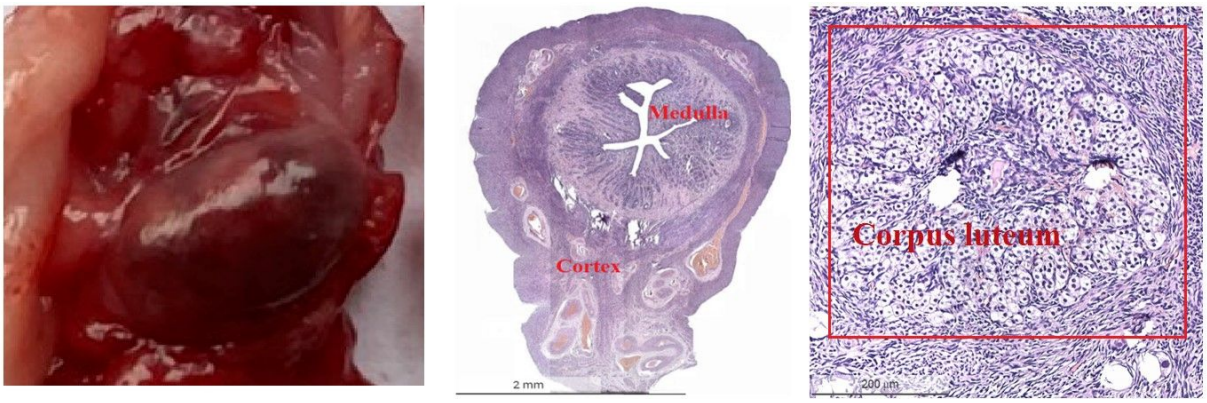
where $\bar{x_1}$ is the mean value for the first set of samples; $\bar{x_2}$ is the mean value for the second set of samples; n_1 is the number of the first set of samples; n_2 is the number of the second set of samples; SD_1 and SD_2 are the standard deviations for the respective sets of samples and are found from the formula (5).

If the absolute value of the test statistics t_s is greater than t_{cd}, which is the critical value of the test (tabular value corresponding to a two-tailed test with significance level ($\alpha = 0.05$)), then the differences of the means are significant.

Results and discussion

Histological examination of the samples (Fig. 4, *a*) revealed the cortex and medulla in the structure of the ovaries (Fig. 4, *b*), as well as the presence of a corpus luteum (Fig. 4, *c*).

The medulla contains many blood vessels and nerve endings. In the cortical layer there are follicles in which eggs are formed and mature [16].



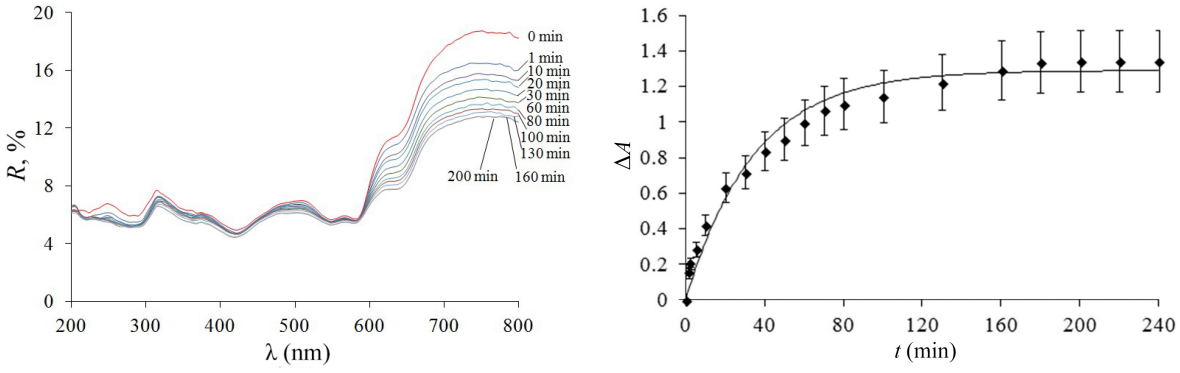
a *b* *c*

Fig. 4. (*a*) Photo of the ovary in the corpus luteum phase, (*b*) histological structure of a section of a cat's ovary (stained with hematoxylin-eosin), and (*c*) corpus luteum of the ovary (color online)

The DRS of samples of cat ovaries, when interacting with glucose, are shown in Fig. 5, *a*. In the UV range, the original DRS spectra of ovarian samples have obvious dips characteristic of the absorption bands of amino acid residues of connective tissue proteins in the form of collagen and reticular fibers, hemoglobin and porphyrins, as a result, the tissues are very opaque in the UV range due to light absorption and very strong scattering. In the region of about 415–420 nm and 540–580 nm, the observed dips correspond to the absorption bands of oxyhemoglobin (415, 542, and 576 nm). Water absorption in the measured range of 200–800 nm is insignificant [2].

The corresponding kinetics of the difference in effective optical densities at the current and initial time $\Delta A(t,\lambda)$ was recorded at 700 and 800 nm and then averaged (see Equation (1)) of the studied ovarian samples during glucose application. According to Equation (3), we find τ (diffusion time) which amounted to (50.4 $\pm$ 1.7) minutes. The average diffusion coefficient for ovarian samples ($n = 5$) was $D = (8.6 \pm 1.4) \cdot 10^{-7}$ cm^2/s. When studying the diffusion of 40%-glucose in colorectal tissues, the diffusion coefficient was found as $D = 5.8 \cdot 10^{-7}$ cm^2/s [17], which is very close to data received in this work and evidently the difference is due to differences in the tissue structure, as colorectal tissue is supposed to be more dense and thus less penetrative to molecule diffusion.

The coefficient of diffusion of glucose into human gingival post-mortem no-fixed tissue when implementing the OC method was determined as $D = (4.1 \pm 0.8) \cdot 10^{-6}$ cm^2/s [18]. Again, the difference between these two tissues can be explained by the features of their structure. For gingival tissue, diffusion is faster due to a well-developed blood capillary network. The diffusion coefficient of glycerol/tissue water in the liver was $D = 8.2 \cdot 10^{7}$ cm^2/s [19]. Some differences in the diffusion rate could be due to differences in movable water content in the particular tissue [6, 10, 17, 19].



a *b*

Fig. 5. (*a*) DRS of cat ovarian tissue samples when immersed in 40%-glucose, (*b*) difference in effective optical densities (experimental data (symbols) and their approximation (solid curve))

The DRS of ovaries of cats at the initial time and at the end of protocol are shown in Fig. 6, *a*. In the entire studied wavelength range from 200 to 800 nm, the diffuse reflectance decreases within 200 minutes of glucose action, which indicates a decrease in scattering and absorption by biological tissue. This effect is explained by two factors: the outflow of intracellular water together with hemoglobin from the tissue due to osmosis and the diffusion of glucose into the tissue. The TTS of the tissue sample at the initial moment and after its immersion for 200 min in glucose are shown in Figs. 6, *b*, *c*. The total transmittance increases over the entire spectral range. In the region from 200 to 600 nm, the absolute value of the total transmittance does not exceed 0.2%, which may be associated with a significant developed ovarian capillary network in the luteal phase.

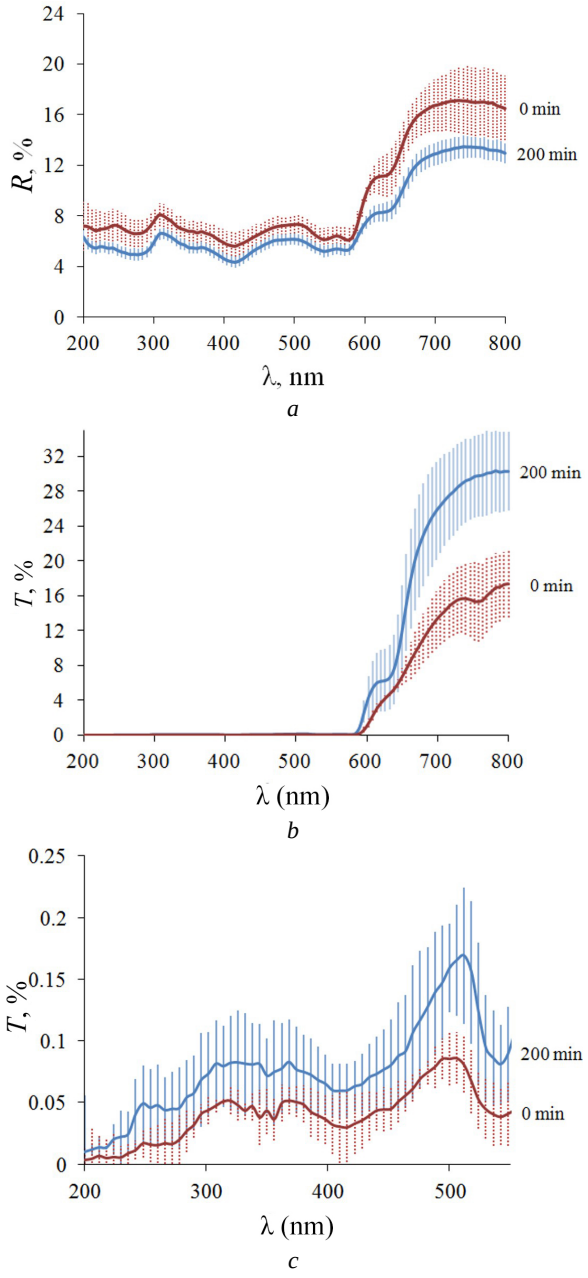


Fig. 6. DRS (*a*) and TTS (*b*, *c*) of cat ovary tissue before and after immersion in glucose in the range from 200 to 800 nm

For the diffuse reflectance spectrum, t_{cd} over the entire wavelength range takes values from 2.36 to 2.57, which is less than $t_{st} = 3.3–8.7$, thus the differences of the means are significant. For the total transmittance spectra in the range from 200 to 490 nm, $t_{cd} = 2.3–2.6$, which is more than $t_{st} = 0.5–2.0$, thus the differences in the means of total transmittance spectra before and after 40%-glucose action are insignificant. In the range of 490–800 nm, $t_{cd} = 2.3–2.8$, which is less than the obtained values $t_{st} = 3.8–7.5$, therefore, the differences in the means are significant. Thus, enough evidence has been obtained to say that there is a statistically significant difference between the means of diffuse reflectance spectra (at 200–800 nm) and total transmittance spectra (at 490–800 nm) before and after exposure to 40%-glucose.

After keeping the samples in 40%-glucose for 200 min, the transmittance increases over the entire wavelength range. The values of the efficiency of OC of the cat ovarian tissue under the influence of 40% glucose, calculated according to Eq. (4), are shown in Fig. 7.

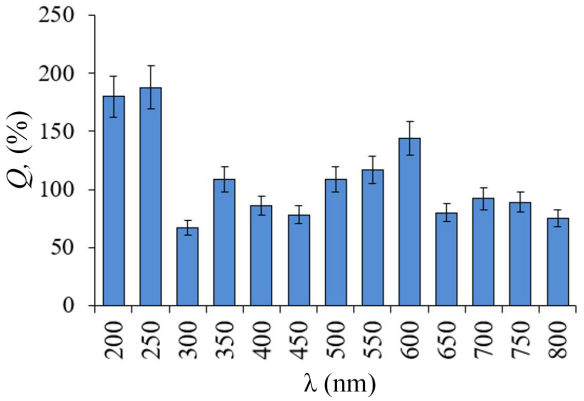


Fig. 7. Efficiency of OC of cat ovarian tissue by immersion in 40%-glucose

In the UV range, the efficiency of OC of cat ovarian tissue with glucose reaches 200%, at 550 nm it reaches 130%, at 600 nm – 150% and 700–800 nm – 80% (see Fig. 7).

Conclusion

The use of optical clearing technology reduces scattering and absorption of light by tissues and, as a result, improves its penetration into deeper tissue structures. The studies have shown that

40%-glucose is an effective OCA for topical use. Quantitative kinetic data of glucose impact will be of interest for further study of the amount of free and bound water in ovarian tissues, in the differentiation of normal and pathological ovarian tissues and in clinical applications.

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Поступила в редакцию 23.12.2022; одобрена после рецензирования 14.03.2023; принята к публикации 20.03.2023
The article was submitted 23.12.2022; approved after reviewing 14.03.2023; accepted for publication 20.03.2023