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# PRECLINICAL TRIALS FOR ADVANCED CHITOSAN-BASED COATINGS IN TREATING PURULENT WOUNDS

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**ABSTRACT** — Wound healing is a complex set of body responses to damaged tissues, which manifests itself through local destructive-inflammatory changes and general reactions. There are numerous coatings used currently in clinical practice to treat wounds. These coatings serve to develop a wet anti-bacterial environment, which is essential to facilitate the healing process. This work focuses on studying multifunctional coatings, which are based on chitosan, a biocompatible polymer featuring wound-healing properties. The distinctive feature to be found in chitosan fibers is their density and the orientation of pores. The coatings within this study were tested on scalped full-layer skin wounds of male Wistar-Kyoto rats and male rabbits. The sample with a dense structure and low biological resorption was found to be effective in performing the function of a framework, as well as in ensuring proper drainage at the affected area. This is important when treating purulent wounds.
The experimental sample with a high degree of adhesion and a shorter biodegradation life can be recommended for treating wounds with no purulent-inflammatory issues, for granulating wounds, as well as a drug carrier-matrix. The studied wound coatings have revealed their medical efficiency at the preclinical stage (in vivo). Using wound coatings with specified structural and functional features would allow making a reasonable choice when selecting a coating depending on the stage of wound healing course.

**KEYWORDS** — chitosan; purulent wound; infected wound; wound process; wound coatings; wound treatment; preclinical tests; regenerative medicine; tissue reconstruction.

## INTRODUCTION

One of the urgent issues faced currently by medicine is the treatment of patients with injuries, burns, and various skin defects (e.g., trophic ulcers, bedsores, infected postoperative wounds, etc.). All this indicates the need to develop highly effective wound-healing medicines with antimicrobial properties [1–3].

The main factors that impede epithelialization and granulation include tissue dystrophy, oxidative damage, wound moisture content imbalance, infections and other complications affecting the area of surgical resection, injury or burn. The development of scar tissue or other structural changes at the wound will reduce the patient's life quality. Nowadays, there are certain techniques available, which can help facilitate the wound healing process as well as improve the structural and functional properties of the newly developing tissue. Most of such techniques involve wound coatings that differ in their composition and functional features [4–6, 25–27].

The data available in respective literature reveals that purulent-inflammatory processes affecting soft tissues are rated among the most common issues in the entire body of surgical morbidity. The pathologies in question account for 35–45% of all surgical cases, while purulent complications, which affect the post-operative wound area, have been observed in 33–38% of all patients. Moreover, as far as outpatient clinical practice is concerned, soft tissue infections constitute the predominant pathology within the total structure of primary surgical patients [7].

The physical methods, which are used to treat wounds nowadays (vacuum treatment of wounds, ultrasonic cavitation, wound treatment with a pulsating jet, etc.) do add to the positive treatment outcome. However, even with all these advanced methods, the treatment of purulent and infected wounds can never be effective without wound dressing [8].

In modern conditions, the physical and hygienic properties featured by materials based on natural cotton no longer meet clinical specialists' needs. Given that, there has been a wider use of dressings made with advanced technologies and featuring a set of the following properties: inertia in relation to biological tissues; minimal mechanical trauma potential; impermeability to microorganisms and dust particles; pH control; gas composition and humidity of the environment around the wound. Therefore, one of the major requirements for advanced wound coverings (dressings) implies their maximum action polyvalence [9–11].

There are over 470 samples of various dressings and wound coverings available now globally and coming in various pharmaceutical forms, whereas the basis for their manufacture includes polymers, collagen, gelatin, cellulose, pectin and many other substances, including various combinations of these materials that feature the respective properties and offer enough pathogenetic grounds to use them to treat wounds [12–14]. This means that one of the promising ways to improve the quality of the treatment offered to patients with infected and purulent wounds implies a scientific and experimental search for new dressings or modifying the already available ones.

Taking into account the above-described requirements for modern wound coatings, one of the most important properties that such materials should possess is biodegradation. From this stance, we believe chitosan — a derivative of the natural polysaccharide chitin — is to deserve special attention [15–18].

The increased interest taken in it seen as a basis for the production of wound coatings can be explained by its capacity to change its physical and chemical properties depending on changes in the molecular weight and three-dimensional structure. At the same time, the form factor of the product can change from a hydrogel to a frame structure with different degrees of density, swelling and moisture absorption. The specific features inherent in this polymer and the materials made on its basis explain the potential for its use. [19].

Such features include minimal side effects, biocompatibility, high healing potential, moisture- and air-permeability, high porosity, mechanical stability combined with plasticity, and a predictable bioresorption period within the body. Chitosan possesses hemostatic, bacteriostatic, and fungistatic properties. There have also been immunomodulating and antitumor effects identified, as well as lack of immunoreactivity has been proven along with complete elimination out of the body and biostimulation of regeneration [20, 21]. The study has also shown that chitosan films can inhibit significantly the growth of microflora (Staphylococcus, Proteus, Pseudomonas aeruginosa), at the same time accelerating the healing of burns. The universal mechanism, which promotes selective binding of chitosan to sugar receptors on the cell membrane, produces a bacteriostatic effect, which, in turn, stops the infectious process involving the most significant microorganisms [22, 23].

In view of all the properties mentioned above, which are featured by the proposed chitosan-based coatings, we can talk about the holding a research project aiming at developing dressings with a polyvalent action range.

### *Aim of Study:*

To expand the range of advanced multifunctional chitosan-based wound coatings with significant antimicrobial and healing properties; to study in vivo the specific features of the wound process involving the treatment of purulent soft tissue wounds of various origins.

## METHODS

### *Study Design*

A multi-stage randomized controlled experimental study involved 225 conventional male rats, weighing 250–300 g each — at Stage 1; 135 individuals of linear Wistar male rats, weighing 250–300 g each — at Stage 2, and 27 male rabbits (Soviet Chinchilla breed), weighing 2,900 (±150) g each — at Stage3 of the study.

To create a model of purulent soft tissue wound in experimental animals, we employed our own technique (Patent for invention RU # 2703709 of August 23, 2018). This comes down to developing a wound featuring specified parameters through inserting a rounded polymer implant into the soft tissues for 6-7 days. Wound infection in animals was modeled through the introduction of Staphylococcus aureus (S. aureus 209P (ATCC6538P)) on foam balls for soft application of liquid and paste-type medicines Pele Tim # 3 (VOCO, Germany). To create infecting, a suspension of an 18–20-hour microorganism culture in a saline solution was used (concentration — $10^5$ microbial bodies per 1 ml). 0.1 ml of bacterial suspension was injected in each ball with a sterile disposable syringe (1 ml) (Beijing Fornurse Medical Equipment Co., Ltd. Ltd, China).

The infecting dosage was 105 CFU of S. aureus 209P per animal. The implant was inserted through a layer-by-layer dissection of soft tissues down to the required depth. Further on, the wound was sutured in layers for the above-mentioned period. The implant was removed surgically, and the tested material was inserted into the resulting cavity. In the control group, the animals did not have the material sample injected into the wound. The tissue defect above the experimental cavity was layer-by-layer sutured with apposition interrupted sutures. The surface area (S) and volume (V) of the cavity were calculated using the following formulas: $S=4\pi r^2$, $V=4/3\pi r^3$, where r is the radius of the implant, $\pi = 3.14$.

Throughout the study, the skin sutures were treated with an antiseptic (the trade name of the antiseptic is not mentioned here thus aiming to avoid a conflict of interest).

Experimental samples of multifunctional chitosan-based wound coatings were developed and synthesized on the basis of the National Research

Center "Kurchatov Institute" (Table 1). The specific issue about the materials was the density and the pore orientation — isotropic or vertical.

*Table 1. Technological features of chitosan samples used through the experiment*

| Sample | Porosity | Wall thickness range | Pore size range | Elasticity modulus | Deformation in compression | Offset yield strength |
|---|---|---|---|---|---|---|
| | [%] | [nm] | [μM] | [MPa] | [%] | [kPa] |
| #1 | 98 | 350-1000 | 20-45 | 0.749 | 44.32 | 29.70 |
| #2 | 98 | 600-1200 | 70-200 | 0.369 | 50.14 | 24.15 |

Table 1 offers a view at the major parameters featured by the samples under study.

All experimental surgical interventions performed on laboratory animals were done in aseptic conditions and under general anesthesia. A combination of drugs (atropine sulfate, prednisone, Sedamidine, Telazol) was used as the anesthetic.

To reduce the general anesthesia time, upon completion of the respective manipulations, the animals were injected with Antimedin. To relieve the pain syndrome within the postoperative period (Day 1), the animals were injected with Flexoprofen (intramuscular).

### Inclusion criteria

For the purposes of the study (through all of its stages), male rats, as well as male rabbits were used with no external signs of diseases and anatomical disorders, that were quarantined in the retainer unit of the academic & production department of the Kuban State Medical University. The study involved animals that developed purulent wounds with standard signs of purulent inflammation during the simulation period.

### Environment of study

Through the entire study, including postoperative periods, the animals were monitored with free access to water and food, in accordance with Russian State Regulatory Standards 33044-2014 "Principles of Good Laboratory Practice". The study was carried out on the premises of the training and production department of the Kuban State Medical University (Krasnodar, Russia).

### Duration of study

Each stage of the study went on for 28 days, taking into account the 7 days required for the wound cavity development. Measurements, diagnostics, and sampling of the material for histomorphological examination at all the stages were done on Days 7, 14, and 21.

### Major study outcomes and registration methods

The main outcome of the study was the identification of the most appropriate sample in terms of its parameters in the experimental groups, as well as the observed differences in the wound process dynamics between the control group and the experimental one.

An additional expected outcome was compliance with the data obtained through non-invasive ultrasound imaging of the wound defect area, in order to study changes in the structure of the tested material samples undergoing biodegradation, as well as the dynamics of changes affecting tissues in the area of the experimental wound.

The study involved analyzing the wound process parameters (reduction of the wound volume, healing rate, sample biodegradation rate, complications, histomorphological composition of the wound) while using various samples of the designed wound coating. Visual assessment of the wound process course included keeping track of the timing of reduction of edema, hyperemia of the tissues around the wounds, as well as any discharge from the wound.

The tissues were subjected to histomorphological assessment following the generally accepted algorithm. The explanted tissues were fixed for 3–5 days in a 10 % neutral formalin solution (Histolab, Sweden), washed in running water for 60 minutes. The materials were processed subject to the standard procedure employing an automatic method on a Leica TP1020 histoprocessor (Germany). The production of paraffin blocks with samples of the studied materials was carried out using a modular installation Leica EG1150H (Germany). A rotary microtome Leica RM2235 (Germany) was used for slicing the preparations, while the obtained sections (thickness — 5 microns) were stained with hematoxylin and eosin according to the standard method. The micro-preparations were studied on an Olympus XH41 microscope (Japan). The morphological evaluation of the created materials structure was done in the Versa 3D SEM/FIB DualBeam scanning election microscope (FEI, USA) with various accelerating voltages, equipped with a Gaseous Second-

ary Electron Detector, a Peltier-cooled table and an option of using the ambient mode.

Ultrasound examination of the tissues was carried out with a Mindray M7 ultrasound scanner, using a high-frequency linear ultrasound sensor L 12-4s (China), with an operating frequency of 6–10 MHz — on the day the surgery was performed and then two days later, until the animal was removed from the experiment, in the following modes: in the color Doppler mapping mode using a pulse-wave Doppler as well as in the constant-wave Doppler mode.

### *Statistical analysis*

At Stage 1, 2 samples of wound covering were examined in different modifications, for which 4 experimental and 1 control groups were formed, 45 animals (conventional male rats) in each group. Within the control term time, 15 animals within each group were removed from the experiment.

Stage 2 involved studying 2 types of wound coating samples of (# 1 and # 2) based on the criteria of porosity, mechanical strength and biodegradation terms. The properties of the samples were studied in 2 experimental and one control groups, each including 45 animals (linear male Wistar rats). Within the control period, 15 animals of each group were removed from the experiment.

At the final stage, male rabbits were used, which were broken into 2 experimental and 1 control groups, 9 animals in each. In each control period, similar to the previous ones, 3 animals of each group were removed from the experiment.

The statistical processing of the study results was done on a personal computer in the Windows 10 operating system using the STATISTICA 6.1 (StatSoft, Inc., USA) and Excel (Microsoft Office 2010) software. The hypothesis of the cumulative distribution normality in the samples was tested relying on the Shapiro-Wilk and Kolmogorov-Smirnov criteria. The statistical significance level was set at $p<0.05$. The differences between the quantitative parameters with a normal distribution were evaluated through the Student's t-test, whereas independent samples were evaluated relying on the nonparametric Mann-Whitney test. The differences in all cases were considered statistically significant at $p<0.05$. The significance level of the relationship between the two qualitative variables was tested through Pearson's Chi-square ($\chi^2$) test.

## RESULTS

### *Major results of study*

Preliminary screening studies carried out at Stage 1 of this experiment revealed 2 most promising types of wound coatings made of modified chitosan, which featured fundamental differences, both physical and chemical. This allows differential change in the tactics of purulent wound local treatment, depending on the specific task.

Stage 2 of the experiment implied studying the samples of wound coatings selected through Stage 1 following the criteria of porosity, mechanical strength and biodegradation time. The properties of the samples were studied in conditions of a purulent wound. The result of the study revealed certain advantages demonstrated by sample # 1. Its biodegradation process was uniform; the distribution in the wound cavity was equal, which contributed to a smooth therapeutic effect in all parts of the wound cavity.

The assessment of the biodegradation degree of the samples studied at Stage 2 in % ratio to the initial volume revealed a statistically significant difference in biodegradation between samples # 1 and # 2. Sample # 2 underwent biodegradation 7 times as fast ($p<0.01$) and by Day 9 was basically undetectable within the operation area (Fig. 1).

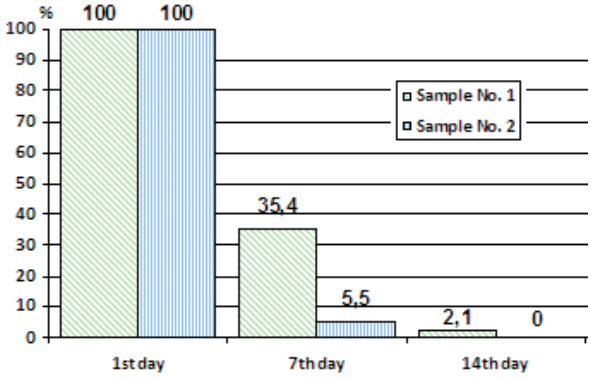
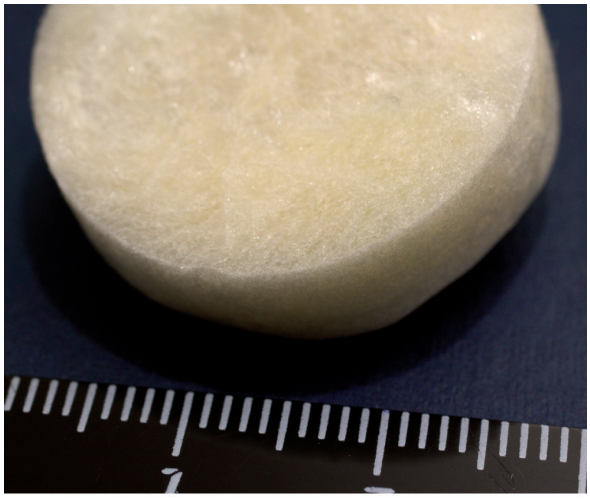


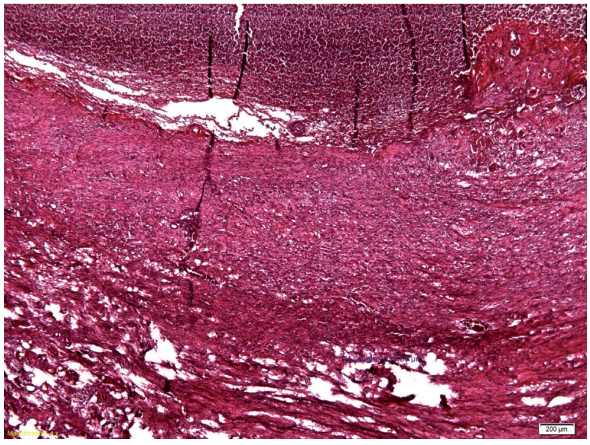

***Fig. 1.*** *Residual wound coverage volume (% of original) in the target time frame – p<0,01.*

The histological representation, following the introduction of a test material sample, is a developed abscess in the muscle tissue, where the walls consist of 2 layers — a pyogenic membrane (up to 0.3 mm) and a developing connective tissue capsule (up to 1.5 mm). The abscess cavity contained a partially biodegraded sample distributed evenly. The muscle tissues surrounding the capsule featured an edema with an increased number of full-blood capillaries, with certain signs of stasis and lymph-macrophage infiltration (Fig. 2).

Stage 3 was carried out in order to study the chitosan-based multifunctional wound coating properties in larger purulent wounds affecting soft tissues.



*a) Appearance of sample No. 3, with a magnification of x4*



*b) Fragments of sample no. 3; homogeneous biodegradation (7 days)*

***Fig. 2.*** *a) — Appearance and histomorphological picture of sample No. 3, with signs of homogeneous biodegradation on the 7th day after its introduction into the experimental wound b) — (1 — homogeneous biodegradation of the material; 2-pyogenic capsule).*

The area chosen to develop the wound cavity was the shoulder-scapular region, whereas the trapezius muscle tissue was chosen as the depth of the simulated cavity.

The polyvalent potential of using the developed samples is due to the experimentally proven properties featured by the selected samples: proper frame function (sufficient density), due bioadhesive capacity, biodegradability and biocompatibility.

All these properties identified in experimental samples were to be found in them in different proportions, which could be accounted for by the specifics of their production technology — the share chitosan in the base solution being the first one, as well as the molecular weight of the sample; features of the pore internal orientation and the ratio of the size and thickness of the walls separating them.

Given that, a programmable change in the initial parameters' ratio of the raw stuff and the use of various options for manufacturing chitosan samples, allow altering the finally obtained properties of the developed wound coatings as listed above.

## Extra results of study

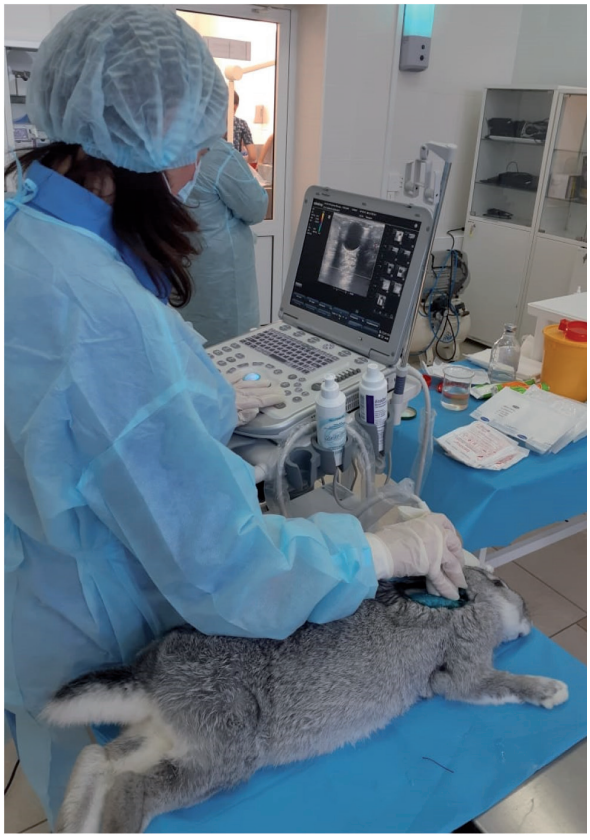
At Stage 3, the experiment was carried out relying on intravital ultrasound of the wound defect area (Fig. 3). The purpose of the ultrasound study was to investigate possible visualization of changes affecting the structure of the test material samples undergoing biodegradation, as well as the dynamics of tissue changes at the experimental wound.

The use of ultrasound to monitor the experimental wounds through the experiment, done in order to ensure a more detailed study of the wound cavity and the surrounding tissues status could be explained by a number of reasons. At the stage of developing the wound cavity, the ultrasound method allowed visualizing the implant inserted into the soft tissues as well as the blood flow in the surrounding tissues, which helped assess the location of the implant in relation to the surrounding anatomical formations, and was of great importance for standardizing the process of the wound cavity development. In view of the dynamics, the ultrasound study allowed identifying the degree of the wound cavity capsule development, any wound discharge inside the cavity, as well as the blood flow status at the surgical intervention area.

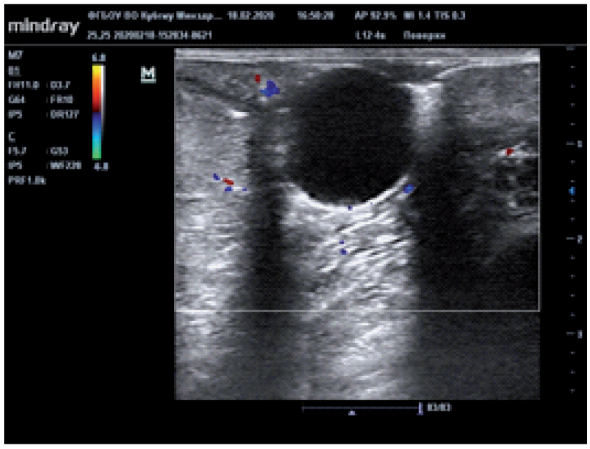
At the further stages of the experiment, the method of ultrasound examination of the experimental wound area containing a coating sample allowed not only visualizing biodegradation of the tested material, yet also identifying the major ultrasound semiotics of soft tissue damage – inflammatory infiltrate at different stages of its progress, muscle fiber imbibition, the appearance of a purulent substrate and signs pointing at the beginning reparation.

The verification of the data obtained through the ultrasound examination was done by studying and comparing the biopsy material obtained when removing the animals from the experiment within the above-described control periods. This method helped us arrive at non-invasive assessment of the wound process status when using the selected samples of wound coatings, as well as it helped visualize the samples of the studied material, which were undergoing biodegradation (Fig. 4).

Following the introduction of chitosan samples into the muscle layer, they were visualized as formations featuring clear and even contours and a homoge-



*a) Conducting an ultrasound scan at the stage of introducing a polymer implant into soft tissues at stage 3 of the experiment*

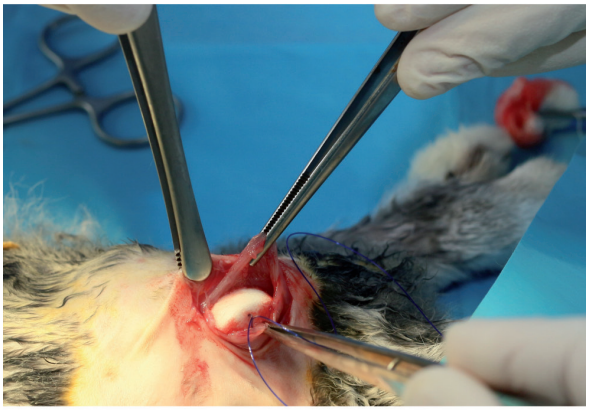



*b) Assessment of the blood supply to the surrounding tissues (1) (2)*

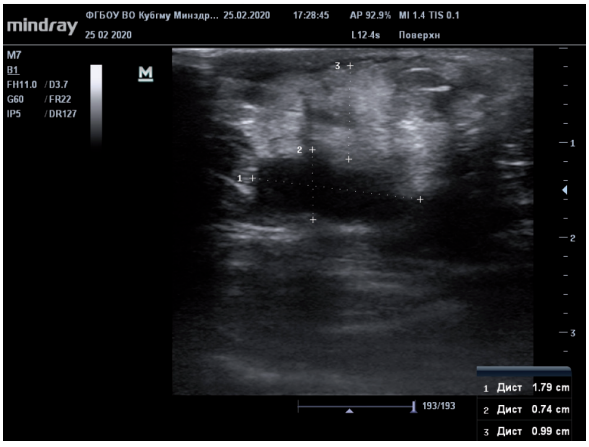
***Fig. 3.*** *a) Ultrasound at the stage of introducing a polymer implant into soft tissues. b) — Assessment of blood supply of tissues (2) surrounding implant (1)*

neous structure with the effect of distal pseudo-reinforcement, the size being up to 20–20–20 mm.

Along the periphery of the implanted sample, there were vital tissues with clear ultrasound structure



*a) Introduction of a sample of the test material (1) into the formed experimental wound*




*b) Ultrasonic location of the sample (1) of the test material in soft tissues*

***Fig. 4.*** *Comparison of a) visual and b) CD images of tissues and samples of the drug, when performing various stages of the study*

and echogenicity visualized. Vascular structures of muscle tissue were visualized as single blood flow loci revealing a spectrum of arterial and venous blood.

By Day 7 into the observation, the size of the wound coating samples regressed. What is more, sample # 2 had nearly completely biodegraded: the size of the wound cavity shrank down to 5–10% of its initial size. In the remaining wound cavity, a small amount of exudate (up to 0.25 ml) was to be observed. During that, the size of sample # 1, which was the denser one, as well as the size of the cavity it was placed in, shrank down to 30–35% of its initial size. The peripheral area of the remaining wound cavity featured denser hyperechogenic and homogeneous layers of muscle fibers of various thicknesses (2.5 mm to 12 mm) appearing there.

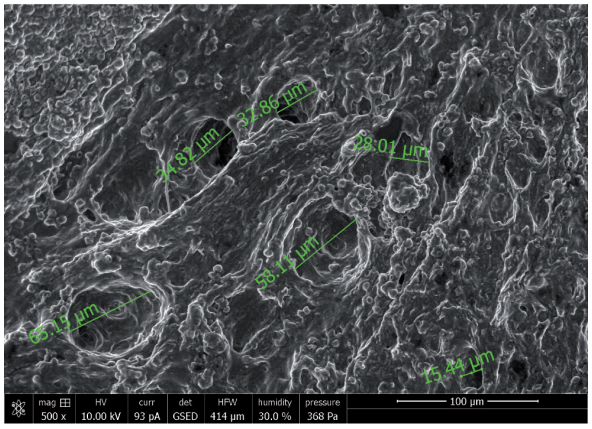
There was also an increase observed in the blood flow speed within the arterial and venous network – Vmax art — 25–30 cm/s; Vmax veins – 15–17 cm/s.

The study conducted on Day 14 into the experiment revealed that infiltrative changes affecting the muscle tissue surrounding the experimental wound cavity featured different stages of inflammation. There were signs of dense and loose infiltrate (layer thickness ranging from 1.7 mm to 9.8 mm) visualized, as well as the muscle fiber imbibition, with an increase in its volume (from 4.4 mm to 12.8 mm). The cavity of the wound defect, where the denser sample # 1 was implemented, decreased to 1.2 x 4.3 x 1.7 mm (H x W x D), with some exudate identified, which manifested itself as anechogenic strips on the periphery of the sample residues (up to 0.5 ml).

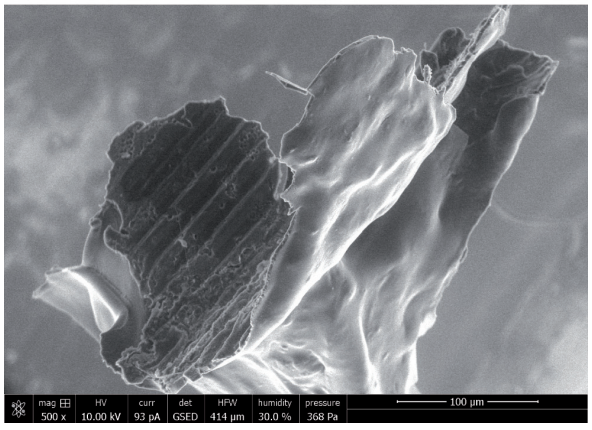
Upon introducing sample # 2, the residual effects in the experimental wound were visualized as loose-fibrous (hyperechogenic) formations with a diameter of up to 2.5 mm, which featured signs of infiltrative changes in the surrounding tissues. The maximum speed of the venous blood flow in the infiltrate zone was Vmax veins — 5 cm/s, which almost matched the initial value.

There was also a clear visualization of the boundaries separating the muscle-aponeurotic layers in the respective area, with a differentiation of a striated muscle tissue pattern.

To further study the tested wound coating sample biodegradation, we relied on the scanning electron microscopy method. On Day 7 into the experiment, samples # 1 were obtained. The result of the study confirmed the theoretical calculation of the step-by-step sample biodegradation with the capillary (sorption) properties preserved (Fig. 5).




*a) Cellular structure preserved in the sample*




*b) Internal — with preserved structure (1) and external — partially biodegraded (2) surfaces of the test material in soft tissues*

***Fig. 5.** Investigation of the biodegradation process by scanning electron microscopy*

### *Adverse events*

No adverse events were observed through the experimental study.

## DISCUSSION

The developed wound coatings featured proper framework functions, as well as due bioadhesive capacity, biodegradability and biocompatibility.

The study results obtained through investigating the properties of the developed chitosan-based wound coatings correlate with the literature data in terms of comparing properties like bioadhesive capacity and biodegradability [21]. The experimental data appear comparable with the results other researchers obtained — there was confirmation obtained for the calculated qualities of the impact that the respective samples had on the surrounding tissues, in view of the specifics of the wound defect occurrence and specific wound healing processes, as well as there was potential identified for ensuring a proper microclimate: controlled pH, humidity; thermal insulation of the wound; creating a barrier against microorganisms; ensuring due sorption activity of the material; atraumatic capacity in terms of granulations [15–17].

A comparison of the developed wound coatings revealed that various synthesis technologies allowed controlling their physical and chemical properties as well as possible use of these coatings within various treatment schemes not only when dealing with issues of infectious origin, yet also when treating other soft tissue lesions.

## CONCLUSION

The chitosan-based wound coatings studied through a multi-stage experiment offered proof to the properties predicted while developing the materials, namely, a stable adhesive capacity to biological tissues and the framework function.

Sample # 1 featured moderate strength, elasticity, as well as a denser structure and, respectively, a lower level of biodegradation (bioresorption on Day 14), which determined its capacity to maintain the desired shape and the initial dimensions for a long time. This, in turn, allowed the sample to effectively perform the framework functions and ensure proper drainage of the pathology focus. The mentioned features revealed that the sample could offer a promising choice when dealing with purulent wounds.

Sample # 2 was of lower strength, elasticity, density, as well as a higher adhesion degree (compared to sample # 1), with complete biodegradation achieved on Day 7. In view of the test sample features, it can prove of the best use in cases with no purulent inflammation and with granulating wounds. Besides, sample # 2 revealed a capacity to transform into a gel with a highly ordered internal structure of the micellar type, which allowed introducing another function – the use as a carrier matrix for the drugs introduced into its structure. This condition will allow expanding the indications for use.

The parallel ultrasound examination technique employed to study experimental wound areas produced an expected positive effect. The match between the data obtained during non-invasive visualization of the analyzed samples and the surrounding tissues, with the data obtained through histomorphological studies allowed reducing both the number of invasive procedures and the number of animals to be involved in the study process.

## ETHICS PRINCIPLES COMPLIANCE

The study described above was granted approval at the meeting of the Independent Ethics Committee, the Kuban State Medical University (Protocol # 63 of May 21, 2018) and was carried out within the period of 2019-2021.

## FUNDING

The authors herewith claim no sponsorship used for the study.

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