Nanostructured Carbon Materials for Biomedical Use

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Abstract

One of the priority trends of carbon nanotechnology is creation of nanocomposite systems. Such carbon nanostructured composites were produced using - raw materials based on the products of agricultural waste, such as grape stones, apricot stones, rice husk. These products have a - wide spectrum of application and can be obtained in large quantities. The Institute of Combustion Problems has carried out the work on synthesis of the nanostructured carbon sorbents for multiple applications including the field of biomedicine. The article presents the data on the synthesis and physico-chemical properties of carbonaceous sorbents using physicochemical methods of investigation: separation and purification of biomolecules; isolation of phytohormone - fusicoccin; adsorbent INGO-1 in the form of an adsorption column for blood detoxification, oral (entero) sorbent - INGO-2; the study of efferent and probiotic properties and sorption activity in regard to the lipopolysaccharide (LPS), new biocomposites - based on carbonized rice husk (CRH) and cellular microorganisms; the use of CRH in wound treatment. A new material for blood detoxication (INGO-1) has been obtained. Adsorption of p-cresyl sulfate and indoxyl sulfate has shown that active carbon adsorbent can remove clinically significant level of p-cresyl sulfate and indoxyl sulfate from human plasma.

Enterosorbent INGO-2 possesses high adsorption activity in relation to Gram-negative bacteria and their endotoxins. INGO-2 slows down the growth of conditionally pathogenic microorganisms, without having a negative effect on bifido and lactobacteria. The use of enterosorbent INGO-2 for sorption therapy may provide a solution to a complex problem - detoxication of the digestive tract and normalization of the intestinal micro ecology.

The immobilized probiotic called "Riso-lact" was registered at the Ministry of Health of the Republic of Kazakhstan as a biologically active food additive. The developed technology is patented and provides production of the medicine in the form of freeze-dried biomass immobilized in vials.

Introduction

At present, a universal trend in therapy and preventive measures against gastric and intestinal diseases is the use of oral (entero)sorbents of different chemical nature which have a high sorption capacity towards binding infectious agents and pathological products of metabolism, thereby exerting an antidote effect. As adsorbing, antidote, and antidiarrheic preparations are widely used in medical practice, creation of their new forms is an important task of medicine. In this regard, the problem of using effective sorbents as a means of delivering medicinal and probiotic preparations is paramount.

Probiotics immobilized on sorbents are more effective than their liquid forms or dry concentrates of probiotic cultures which are widely used at present [1]. The advantage of such biocomposites is a synergetic action of microbial (antagonistic activity in relation to pathogenic microflora and a proteolytic action) and adsorptive components. As an example of practical usage of this approach we consider the following preparations: bifidumbacterine forte (bifidobacteria immobilized on activated carbon and freeze-dried), lithovite C (a matrix made of zeolite and treated with wheat and rye bran with immobilized bifidobacteria) for the treatment and prophylaxis of dysbacteriosis, as well as vetosporine-active and Vetosporine-Forte based on the biomass of bacteria *Bacillus subtilis* 11B and *Bacilluss subtilis* 12

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B adsorbed on particles of activated coal and zeolite, respectively [2].

In the last decade, the process of vulnerosorption, i.e. removal of toxic components via wound surface or inflammation hotbeds by means of acceleration of their transportation from blood followed by their fixation on the sorbent, has been actively used for successful treatment of festering wounds. At present, gelated agents based on polyvinyl alcohol (Ghelevin, Kollavin, lisosorb, Russia); polyacrylate (Tender Wet, Germany); finely dispersed carboxymethylcellulose(CMC); hydrogel coatings, dextrane preparations, oxidized cellulose and its modifications are known. The use of sorbents is expedient, when treating festering wounds during inflammation period, when it is necessary to prevent the action of exo- and endotoxins, proteolytic enzymes of pathogenic bacteria on tissues and penetration of microbial cells themselves inside the wound [3].

When solving the problem of treating festering-necrotic wounds, an extremely promising trend is the construction of new types of nanobiocomposites which combine excellent adsorbing properties of nanostructured materials and a high biological activity of probiotic microorganisms immobilized on them. The use of a low cost sorption material obtained by high temperature carbonization of secondary vegetable raw materials such as rice husk (CRH), which are functionalized by the cells of probiotic microorganisms, is of great interest. It allows the properties of an adsorbent and an active biological agent to combine in a synergetic action.

Another trend of an efferent therapy which is of current interest is the development of new sorbents for extracorporeal detoxication including the treatment of endotoxemia. This is due to the increase in the number of patients with serious diseases caused by endotoxins of gram-negative bacteria such as unsecretable thermostable lipopolysaccharides (LPS) - the main component of the external membrane of the cell wall of these bacteria. The release of endotoxins into the body takes place due to destruction of pathogenic microorganisms causing generalized infections which can result in endotoxic or septic shock and multi-organ failure. Despite the progress recently achieved in understanding of pathophysiological mechanisms of sepsis and a significant improvement of antimicrobial therapy, the mortality rate owing to septic shock is still very high all over the world [4].

Nanoporous Carbon Sorbent for the Molecular-sieve Chromatography of Biomolecules

In the world market there are no sorbents useful for large scale purification of biomolecules at present. The existing sorbents are extremely expensive, their cost varies from a few hundred to a few thousand dollars per kilogram and they are intended mostly for analytical purposes. These sorbents are mainly made of polysaccharide polymers such as dextran or agarose gels. They have very low mechanical strength. Such gels are easily attacked by fungi and microbes, therefore all these sorbents have short shelf life and the term of their exploitation does not exceed a few months. They are not very useful for separation of substances in preparative amounts. Therefore, there is an urgent need, based on the principles of nanotechnology to create a fundamentally new generation of sorbents designed to purify biomolecules and biological structures [5, 6].

Figure 1 shows the image of a particle surface of carbon produced from the apricot stones by carbonization at 800°C. For the analysis, the particulate samples ground to 1 mm were taken. The micrograph, shows the presence of pores of 200 nm diameter and less at the internal surface of larger pores.

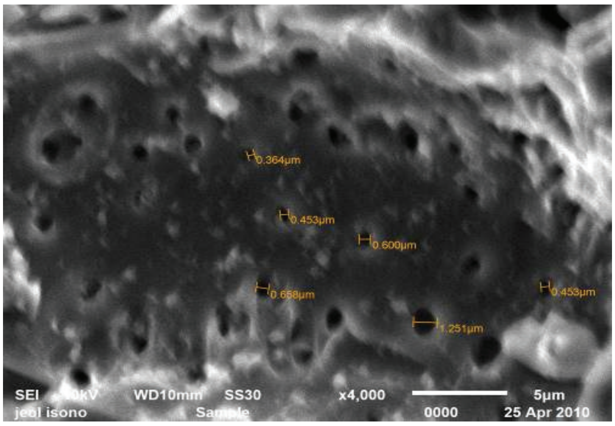

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Fig. 1. Electron micrograph of a particles surface of the sample carbonized at 800 °C.

The next task of the research, was the use of the carbon sorbent for purification of a large albumin - lipid complex (ALC) which is a subcellular organoid of a vegetable cell - spherosome. As ALC are rounded small bodies with the diameter of ~1 µm, for its purification a sorbent with very large pores is necessary.

For the preparation of noncellular extract, 6 g of dry seeds of wheat of the variety «Vitreous-24» was carefully placed in a porcelain mortar in a 0.05 M of tris - HCl buffer, pH 7.4 and ground. The obtained homogenate was centrifuged at 10000 g during 10 minutes. Then the noncellular extract was fractionated on a column with a carbon sorbent. The results of fractionation are presented in Fig. 2.

It is evident from Fig. 2, that ALC is eluted in the first high molecular peak. And all other substances with lower molecular mass are eluted in the second peak. The results obtained testify for the applicability of this carbon sorbent for the molecular-sieve chromatography of large albumin lipid complexes, such as spherosomes.

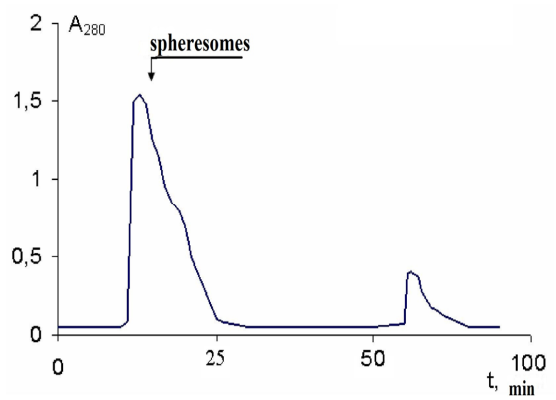

Fig. 2. Molecular-sieve chromatogram of ALC from wheat grain on a column with a carbon sorbent.

Technology for Production of Fusicoccin-Containing Components using Nanocarbon Sorbents

Fusicoccin was discovered in 1964 by the Italian scientist Alessandro Ballio as phytotoxin of pathogenic fungus *Fusicoccum amygdali* Del.[7]. Recently it has been discovered that the phytohormone fusicoccin in nanogram concentrations is needed to treat cancer cells in the state of apoptosis [8].

The Institute of Combustion Problems has developed a method for production of fusicoccin extract from germinated wheat seeds, which is based on the selective sorption of fusicoccin by a nanostructured carbon sorbent [9].

The study of the cytotoxic activity of fusicoccin extract. The objects of the study were three samples analyzed for the presence of substances with cytotoxic activity against larvae of marine crustaceans *Artemia salina* (Leach) under culture conditions *in vitro* [10, 11]. Statistical analysis was carried out using the computer program FNI. The results of the study on cytotoxic activity of the tested samples A0, A1, A2 against larvae of *Artemia salina* are presented in Table 1.

LD_{50} (abbreviation for "lethal dose, 50%"), LC_{50} (lethal concentration, 50%) or LCt_{50} (lethal concentration & time) of a toxin, radiation, or pathogen is the dose required to kill half of the tested population after a specified test duration.

In the study of the cytotoxic activity of fusicoccin-containing extracts it has been found that the extract fractions from the preparative separation (A1 and A2) exhibit significantly higher cytotoxic activity against larvae of *Artemia salina* then the crude extract A0.

Thus, this substrate has a potential for creating an effective anticancer drug.

Table 1

The cytotoxic activity of samples A0, A1 and A2 against larvae of *Artemia salina* under culture conditions *in vitro*

Identification of the substance	concentration ug/ml	The number of surviving larvae (*Artemia salina*)			LD_{50} ug/ml	activity Yes/No
		1-paraallel	2-parallel	3-paraallel		
A0 crude extract	1	10	10	10	140.39	Yes
	10	10	7	9		
	100	7	6	4		
A1 fraction after preparative separation	1	10	10	10	39.22	Yes
	10	6	6	8		
	100	4	3	4		
A2 fraction after preparative separation	1	9	10	9	32.62	Yes
	10	7	7	6		
	100	4	3	3		

Development of a Material for Blood Detoxification (INGO-1)

We have developed a product INGO-1 Sterile, using a single column with the sorbent to purify the blood from toxins. The novelty lies in the design of the column as well as the properties of the sorbent. The main advantages of our columns are: the column is made according to the technology, which allows preventing dust generation in the process of sorption, the column is provided with a built in filter to prevent contact of dispersed particles with the blood, and it provides universal connection to most devices for hemodialysis and hemosorption. Rice husk (RH) is a widespread biomass. Every year around the world more than 120 million tons of this plant material are produced. Creating functional adsorbents based on RH is economically beneficial, and at the same time it solves the problem of waste disposal. In particular, adsorbents based on waste material are products with a high added value. Similarly, purification of blood by hemodialysis is a highly inefficient method of eliminating protein-bound uremic toxins, such as protein conjugates of indoxyl sulfate (IS) and para-cresyl sulfate (PCS), as well as inflammatory cytokines, particularly interleukin IL6 and IL8, which are present in elevated concentrations in the blood of parents with common diseases such as chronic kidney failure (CKD), leading to further violations of the cardiovascular, renal and bone physiology [12]. Sorption therapy that combines the use of carbon nanosorbent with porosity can be used to effectively eliminate those molecules that are difficult to remove by current hemodialysis treatment, and thus can prevent the poor prognosis of patients with CKD [13]. Further in Fig. 3 the scheme of preparation of INGO-1, pore structure formation in the process of activation texture and adsorption characteristics of the samples are presented.

$$K_2CO_3 + SiO_2 \rightarrow K_2SiO_3 + CO_2 \uparrow$$
$$K_2CO_3 + 2C \rightarrow 2K + 2CO_2 \uparrow$$

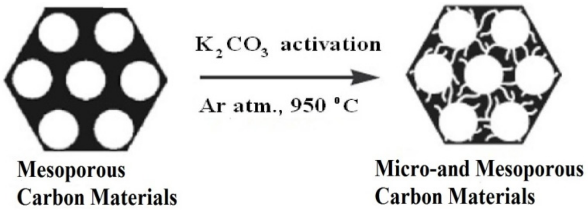

Fig. 3. Pore formation in the process of activation [14].

Physical and chemical properties of the carbon nanostructured materials were studied for INGO-1. In Table 2 the data of mercury porosimetry are presented.

Table 2

Characteristics of the pore structure of carbonized rice husk (CRH) samples obtained with mercury porosimetry

Sample	Time, h	Ratio K_2CO_3/RSH mmol/g	S_{BET} m^2/g	S_{DFT} m^2/g	V_{DFT} cm^3/g	D_{pore} nm
CRH-K_2CO_3 #1	0.5	36	1272	1182	1.6	4.0
CRH-K_2CO_3 #2	2	36	691	480	1.2	4.0
CRH-K_2CO_3 #3	0.5	45	1655	1178	1.0	0.6
CRH-K_2CO_3 #4	2	45	552	366	0.6	3.3

The samples activated for 0.5 hours (CRH-K_2CO_3 # 1 and 3) had a higher surface area as compared to the samples that had been activated for 2 hours. Iodine number values were close to those of the BET specific surface area. Large volumes of mesopores measured by mercury porosimetry and large BET surface area correspond to the maximum adsorption capacity according to the Langmuir model.

In the laboratory of cellular biology and microbiology of Aachen University of Applied Sciences (Germany), we conducted research on the development of the methods for elimination of endotoxin from biological liquids using CRH. The concentration of lipopolysaccharide in the medium was determined photometrically using a directional set (Switzerland) and photometric scanner (Bio-Rad Co., USA).

Figure 4 shows the results of determination of adsorption activity of CRH in regard to LPS. During the first 10 minutes the process of LPS binding proceeds actively and the concentration of LPS in solution decreases by 90%. After 40 minutes LPS in the solution is not detected. In subsequent experiments, when studying the processes of LPS sorption from solutions in the presence of blood proteins albumin, haemoglobin, fibrinogen and lysozyme, it was found these proteins do not prevent the sorption of LPS. It means that carbonized rice husk with a nanostructured surface is a promising sorbent for selective sorption of LPS from different solutions and can serve as a basis for creation of the methods of elimination of endotoxin LPS from biological liquids.

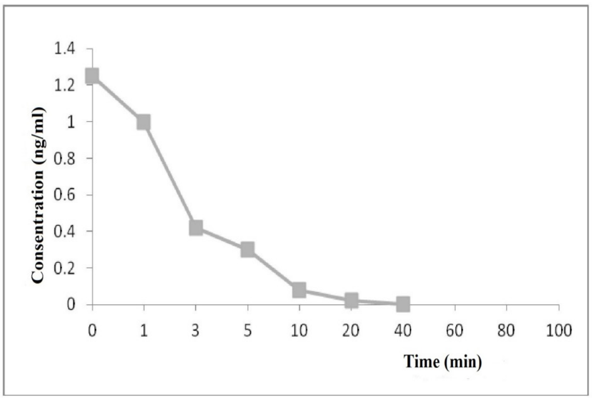

Fig. 4. Dynamics of adsorption of LPS on CRH.

Development of Carbon Enterosorbent INGO-2

One of the topical problems in the field of enterosorption is the search for the materials convenient in use, possessing high sorption properties for a wide range of toxic substances and microorganisms. The use of enterosorbents may provide a solution to the complex problem of detoxication of the gastrointestinal tract and normalization of the microbial population of intestines.

Sorption activity against microbial cells of enterosorbent INGO-2 and the conventional medical enterosorbent - activated carbon (AC) "Carbolong" are given in Table 3.

Table 3
Efficiency of sorption of Gram-negative and Gram-positive bacteria on INGO-2 and the AC

Strains of microorganisms	The initial concentration of cells ×109/ml	Adsorption capacity (10^8 cells per 1 g of sorbent)	
		INGO-2	AC
Gram+ bacteria			
L. plantarum AB-5	1.3±0.04	6.1±0.6	5.6±0.2
L. brevis AB-4	2.4±0.6	8.6±0.3	9.1±0.8
B. bifidum C-16	1.7±0.2	9.1±0.4	7.7±0.1
S. aureus S60	1.8±0.2	9.2±0.3	7.5±0.1
Gram- bacteria			
S. sonnei 158	2.5±0.4	62.1±0.9	8.5±0.4
S. typhimurium T1	2.0±0.3	73.4±0.5	9.4±0.6
E. cloacae 12	2.3±0.08	84.1±0.8	9.9±0.9

Study of sorption efficiency of microorganisms has shown that, firstly, enterosorbent INGO-2 possesses higher sorption characteristics for microbial cells than AC. Secondly, which is particularly important, the sorption capacity of INGO-2 for the gram-negative bacterial cells are much higher than its sorption capacity for the gram-positive bacterial cells. The information obtained on the sorption properties of INGO-2 with respect to Gram-negative bacteria demonstrates the feasibility of application of this sorbent for normalization of microbial flora in dysbacteriosis associated with the increase in population level of Gram-negative enterobacteria.

According to the results of the experiments carried out on laboratory animals which were treated with INGO-2 *per os*, this sorbent has an ability to selectively affect microbial growth, inhibit the growth of opportunistic microorganisms, but without compromising the growth of probiotic microorganisms. The experimental results indicate that INGO-2 in low concentrations (0.005 mg/ml) has a significant positive effect on the growth of intestinal epithelial cells in rats.

The process of preparing enterosorbent INGO-2 is patented [15, 16]. This suggests the possibility of using CRH for immobilization of probiotic bacteria on the surface of cells.

Development of a Sorbed Probiotic

Immobilized probiotics are living microorganisms of a natural microbial flora of warm-blooded organisms or their active metabolites, artificially connected with an insoluble carrier without changing their catalytic properties [17]. In the development of the probiotic sorbed on CRH as a biological component, strains of bacteria of *Lactobacillus* genus were used, referring to the three most common types of intestinal lactoflora: *L.fermentum* AK-2R, *L.acidophilus* AA-1 and *L.plantarum* AP-1 [18].

Immobilization of the bacteria was carried out within 2 hours, then the granules of CRH were washed with an isotonic solution to remove the weakly adsorbed cells, and were used in further experiments.

The electron microscopic study has shown that the cells of *Lactobacillus* form microcolonies on the surface of the sorbent (Fig. 5).

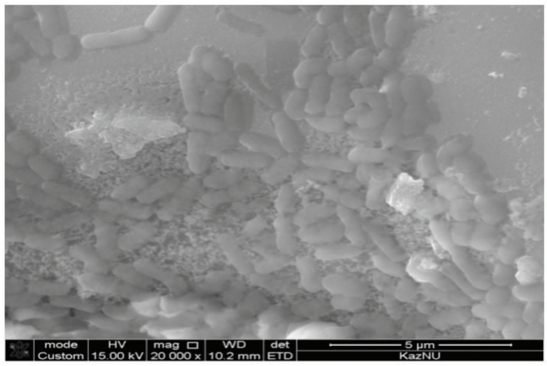

Fig. 5. Electron micrograph of the surface of CRH with cells of a mixed culture of *Lactobacilli*.

The effectiveness of the sorbed probiotic was determined by the parameters that provide its long-term functional activity. These parameters are as follows:
1) increased resistance of cells attached to the sorbent bacteria in acidic medium of the stomach;
2) increased antagonistic activity;
3) engraftment in the colon.

The use of immobilized cells of *Lactobacilli* in experiments with a "model stomach" (incubation of bacteria in the medium with gastric juice) has shown that their viability decreases only by one order of magnitude. The titer of free cells after treatment with gastric juice falls 10.000 fold. As the most important characteristic of the efficiency of probiotic action is their antimicrobial activity, it seems appropriate to study the influence of immobilization on this indicator after co-cultivation of the immobilized cells of Lactobacilli (ICL) in a MRS-5 liquid nutrient medium for 24 hours with cells of three test-cultures: *Salmonella typhimurium*, *Staphylococcus aureus* and *Candida albicans* (Table 4). The inhibition effect of a sorbed probiotic was determined by the quantity of the survived cells of test strains (% to their level at cultivation in a nutrient medium without probiotics). After 24 hours of co-cultivation target microorganisms of cells with a composite material sorbent + probiotic (ICL), only 8.3% cells of yeast and less than 15 of rest bacteria remained alive.

Table 4
The antibacterial activity of the sorbed probiotic by co-cultivation with target microorganisms

Option	Number of viable cells of test strains, % control		
	Salmonella typhimurium 50-90	*Staphylococcus aureus S60*	*Candida albicans KAA88*
ICL	0.8±0.01	0.6±0.03	8.3±0.2
FCC	17.5±2.1	16.5±1.3	29.6±1.6
CRH	67±2.6	70±4.6	72±5.3
Control	100	100	100

Note: ICL - a complex of immobilized cells of lactobacilli, FCC-free complex cells; CRH - carbonized rice husks.

An effective probiotic action of *Lactobacilli*, immobilized on CRH shown by experiments carried out *in vitro*, was also evaluated in experiments *in vivo*, by studying the engraftment(incorporation of grafted tissue into the body of the host) ability of exogenous strains. The clinical efficacy of probiotics is associated with the colonization of the intestinal mucosa and substitutionary restoring of functions of the normal indigenous flora. Although bacterial strains used in the production of probiotics, are usually selected from the microflora of the gastrointestinal tract of human, they still do not possess long colonization resistance of the intestine and are eliminated within 3-5 weeks [19, 20]. That is, the microorganisms that make up the common probiotics perform the function of temporary filling of ecological niche of microbial flora, and for this reason the replacement therapy should be carried out for a long time.

It has been found out that, when taking both free and immobilized cells of *Lactobacillus fermentum* AK-2R, they are detected in the feces on the second day, reaching the maximum on the third day. However, after stopping taking non-immobilized probiotic the concentration of exogenous *Lactobacilli* in the feces decreased up to complete disappearance within 15 days [21].

The data obtained provide evidence that injection of ICL leads only to a transient colonization of intestines by exogenous *Lactobacilli*. At the same time, after the injection of the adsorbed probiotic, a colony of this strain was found even 90 days later after the end of production. It means that settling of mucus of intestine by exogenous probiotics is higher at their introduction in the host organism in the form of the microcolonies immobilized on sorbents.

This assumption found confirmation in experiments on studying the influence of CRH on adhesion of cells of lactobacteria *Lactobacillus fermentum* AK-2R to the epitelium of rats' intestine cells. A positive correlation between concentration of a biosorbent and amount of the adsorbed cells of lactobacilli was found.

In a series of experiments with laboratory animals outbreed rats were used for induction dysbacteriosis by intragastric injection of the ampiox antibiotic in the dose of 40 mg per day within 5 days. The probiotic therapy with the help of both free and immobilized cells of lactobacilli led to normalization of microbiocenosis of experimental animals, however, when using a sorbed probiotic this effect was more pronounced.

We believe that the increased therapeutic effect of the immobilized probiotic is related to the fact that, first, lactobacilli cells attached to the career do not die in the upper digestive tract and fully reach the colon. Second, the existence of lactobacilli on the surface of the sorbent in the form of microcolonies causes stimulation of their production of antimicrobial substances, due to their induction of "quorum sensing." Third, the presence of probiotic micro-colonies provides local colonization of their mucous membranes, resulting in a faster recovery of normal flora and speeding up the repairing processes in the intestinal mucosa.

The immobilized probiotic called "Riso-lact" was registered in the Ministry of Health of the Republic of Kazakhstan as a biologically active food additive. The developed technology is patented and provides the production of the medicine in the form of freeze-dried biomass immobilized in vials [22].

The Use of CRH in Wound Treatment

One third of surgical patients are patients with festering-inflammatory diseases. The procedure of vulnerosorption is expedient, i.e. removal of toxic components through surface or hearth of inflammation and acceleration of transportation of toxic substances from blood by their binding with a sorbent. Due to the development of such an approach,a new trend of efferent therapy was formed in medicine, which is based on use of adsorbent application [3]. As medical practice showed, almost all carbon materials for medical use possess significant adsorption properties in relation to exudate, including the microbes and products of their metabolism, causing the acceleration of clearing of wound and activation of the regeneration processes on the surface [23].

During the study of CRH influence on cicatrization of wounds in the experiments *in vivo* 15 males of outbred white rats (weight 200-230 g) were used. The upper part of the back of rats after capillurgy was treated with ethanol at 70°. Then, on the skin on the left and right sides an incision 2 cm long and 4 mm deep was made by a medical scalpel. During the experiment the rats were kept in separate cages on the standard ration of feeding to complete cicatrization of wounds. Efficiency of the treatment was estimated by the speed of disappearance of basic symptoms (cut short of edema, necrosis, pus, and symptoms of intoxication).

It was found that for the animals from experimental group after the treatment of wounds with the powder form of CRH the necrotic tissues grew into a pappy mass and were easily removed from the surface of wound (Fig. 6).

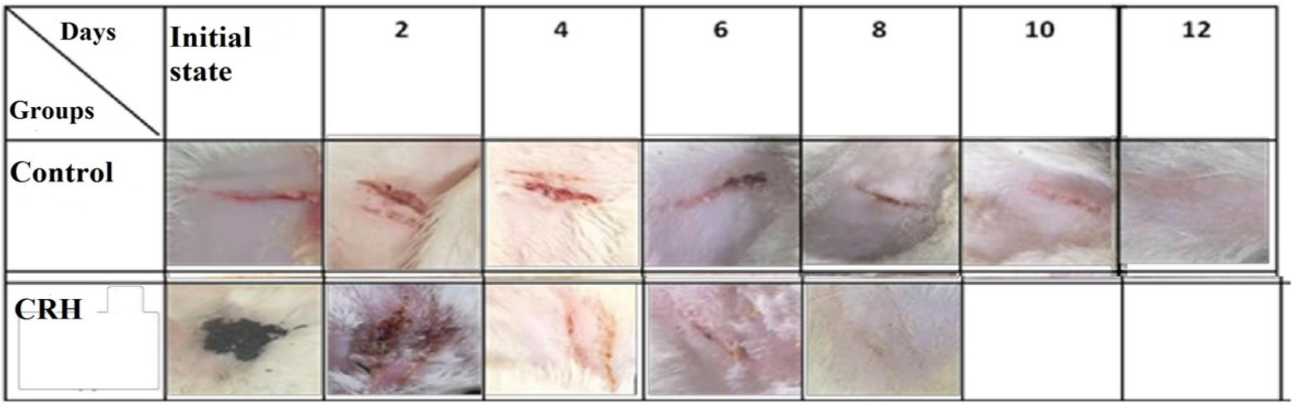

Fig. 6. Dynamics of cicatrization of wounds.

After 12 days' observation it was found out,- that the wounds,- treated with CRH, healed on the 8th day (its length after 4 days decreased from 2 cm to 1 cm, and after 6 days to 0.5 cm). Thus there was reduction of the amount of the wound exudate, the edema of tissue round wounds and their gap decreased considerably. For control animals cicatrization took place only on the 12th day.

In subsequent experiments, the action of biocomposites on the festering wound infected by staphylococcus was studied. It was found that the use of carbonized sorbent notably inhibits the development of festering infection on skin, there by reducing the risk of generalization of infection.

A high healing effect of CRH in regard to cicatrization of festering wounds can be explained by the presence of a great amount of macro, meso and micropores on its surface that participate in the processes of sorption from the wound surface of microorganisms and components of festering exudate.

Conclusions

1. Carbon materials synthesized from plant raw materials have been obtained. Their characteristics have been studied by a number of physical and chemical methods of investigation: mercury porosimetry, SEM. According to the studies, the obtained carbon materials have a potential as a component of sorbents to clean the blood by hemoperfusion.
2. A new material for blood detoxication (INGO-1) has been obtained. Adsorption of p-cresyl sulfate and indoxyl sulfate has shown that active carbon adsorbent can remove clinically relevant level of p-cresyl sulfate and indoxyl sulfate from human plasma. The results indicated that the produced carbon adsorbents may be useful for treating chronic kidney disease.
3. Enterosorbent INGO-2 possesses high adsorption capacity towards Gram-negative bacteria and their endotoxicity. INGO-2 slows down the

growth of conditionally-pathogenic microorganisms, without causing a negative effect on bifido- and lactobacteria. The use of enterosorbents INGO-2 as the sorption therapy may provide a solution to a complex problem - detoxication of the digestive tract and the normalization of the intestinal micro-ecology.

4. Lactobacteria cells were attached to the surface of carbonized sorbent for creation of probiotic preparations. High clinical efficiency of adsorbed probiotics has been stated; the advantage of this biocomposite is synergic microbial (antagonistic activity) and adsorptive (detoxifying and cells protective action of microbes-antagonists) constituents.
5. The use of sorbent of CRH on the wound surface accelerates their cicatrization, reduces an edema, formation of pus and necrosis.

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