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THE MECHANISM OF CLOTS FORMATION IN BLOOD PLASMA UNDER THE ACTION OF CHITIN DERIVATIVES

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The aim of the research was to find out the clot formation by the action of chitin derivates. The biochemical and immunologic investigation methods such as obtaining of fibrinogen, chitosan derivates, electrophoresis in PAAG, Western blot analysis, ELISE, the activated partial thromboplastin time (APTT) and prothrombin time (PT) coagulological tests were used in these studies. The next results were obtained: chitin derivatives in equal measure cause the clot formation in whole blood, blood plasma and fibrinogen solution; the fibrinogen precipitate formed as a result of their action, practically does not contain fibrin; chitosan does not cause the activation of coagulation factors and the absence of newly-formed fibrin confirms it; the addition of calcium chloride to fibrinogen solution in concentration-dependent manner inhibits effect of chitosan.

Thus, under the action of chitosan, a fibrinogen precipitate forms due to the destabilization of its molecule by the lack of calcium. Absence of fibrin degradation products excludes the possibility of the fibrinolytic system activation and physiological degradation of the clot. It makes no sense to use haemostatic drugs based on chitosan in clinical practice.

Key words: chitin and its derivates, fibrinogen, haemostasis system.

The interest to the chitin and its derivates was increased significantly when it was shown its ability to cause the clot formation. It was about the ability of water-soluble derivatives, primarily chitosan, to stop bleeding quickly even in the case of the arteries rupture [1–4]. Chitosan-based materials have recently been actively promoted to clinical practice as hemostatic agents, although the discussion about the mechanism of chitosan action on the blood coagulation system is continue [5–8]. There are a data, which show that a clot formed by chitosan is unstable to the osmotic pressure of the medium [9]. Such property of the clot gives an opportunity to assume that there is no activation of the coagulation system, but the aggregates of fibrinogen appear because of change of its conformation as a result of chitosan action [10]. An indirect confirmation of this assumption can be considered the rate of chitosan-based applicators action, namely, the practically lightning reaction of the blood to the applicator, which does not allow time for the activation of coagulation factors. In order to verify this assumption, the aim of this study was the investigation of the effect of chitosan and its derivatives — carboxymethyl chitin and lactate chitosan — on whole blood, blood plasma and fibrinogen. Such an approach made it possible to determine the degree of involvement of various systems of regulation of blood coagulation to form a clot by the chitosan action.

Materials and Methods

The following materials and reagents were used in the work: acrylamide, bis-acrylamide (“Sigma”, USA), DS-Na (“Bio-Rad

Laboratories", USA); mixture of proteins — molecular weight markers for electrophoresis Low Molecular Weight Calibration Kit ("Fermentas UAB", Lithuania); horseradish peroxidase ("Sigma", USA); chromogenic substrate S2238 (thrombin chromogenic substrate HD-Phe-Pip-Arg-pNA.2HCl, "GE-Healthcare", USA); BCN-Sepharose 4B ("Amersham Bioscience, Sweden); tris (Dia-M, Russia); other used reagents are national production; Cumassi R-250 (Sigma-Aldrich, USA); thromboplastin, reagent APTT (Renam, Russia).

The fibrinogen purification. Fibrinogen (98% of clotting) was obtained from bovine blood plasma with 16% sodium sulfate salting with subsequent separation of cryofibrinogen according to Varetska method [11]. The specific absorption coefficient of fibrinogen in a neutral medium is 15,06.

Fibrinogen (~30 mg/ml) stored at −20 °C. The purity of the preparate was by electrophoresis method.

Methods of determination fibrin contamination in fibrinogen preparations. Contamination of fibrinogen by fibrin was determine by immunoenzyme analysis with the monoclonal antibodies 1−5A and 1−3 c to alpha-chain fibrinogen/fibrin and to fibrin correspondently, and chromatographically. Sepharose 4B with D-dimer immobilized was used in chromatography for contaminates determination. The fibrinogen was applied to sorbent with 0,05 M Tris-HCl buffer, pH 7,4 with 0,2 M 6-aminohexane acid and 10^{-3} M $CaCl_2$. The elution was carried out by the same buffer, the fibrinogen was eluated with a void volume, and fibrin bound with sepharose.

Preparation of chitin derivatives. Chitin was obtained from *Hermetia illucens* puparia as with the following demineralization: 1% HCl treatment for 1 hour and 2% NaOH deproteinization for 2 hours at temperatures of the room [12]. Carboxymethyl chitin and carboxymethyl chitosan were obtained according to the [13]. Chitosan was obtained according to the [14]. Lactate chitosan was obtained according to the [15].

Blood collection. Donor blood plasma (10 people) was obtained at the Blood Center of the National Military Medical Clinical Center "Main Military Clinical Hospital".

Blood plasma stored at −20 °C not more than 2 months. Blood plasma was defrozen for a water bath at 37 °C, then it was mixed carefully by turning over of closed tube several times. There is no activation of coagulation factors V and VIII for this method of defrosen [16].

The bovine blood (farm of Belotserkovsky National Agrarian University, 2 animals) collected from carotid artery and mixed with an anticoagulant, which is a mixture of 0.12 M potassium oxalate, 0.06 M oxalate, 0.006 M CIT, 0.2 M 6-aminohexane acid on the basis of 65 ml of anticoagulant per 1 liter of blood in polyethylene tube. The tube was close tightly and mixed (not shaked). The blood sample was used for the experiment as soon as it was collected. During the experiment blood was kept at a temperature of 4 °C.

Electrophoresis in PAAG. Electrophoresis in polyacrylamide gel (PAAG) in the presence of DS-Na was carried out by Lemmly method [17] with tris-glycine system for vertical electrophoresis (BioRad, USA) in plates 7−10% of gel with thickness of 0.75 mm.

A sample buffer containing 5% sucrose or glycerol, 2% DS-Na and bromophenol for coloring was added to prepare the samples to a protein concentration of 1 mg/ml. Samples were treated with 5% beta-mercaptoethanol for 10 min at 60 °C to restore disulfide bonds. The electric current for a concentrating gel is 19 mA, and for distributive — 35 mA. The gel was stained with 0.125% Kumasi G-250 solution containing 25% isopropanol and 10% acetic acid. As markers used a Low Molecular Weight Calibration Kit (Fermentas UAB, Lithuania).

Immunoenzyme analysis (ELISA) was carried out in microplates with sorption capacity according to the standard method for soluble proteins. The principle of the method is the specific interaction of the antigen and antibody, the formation of the Ag/At complex and the conjugate and the determination of the complex formed with the substrate mixture by the degree of coloration. To confirm the presence of fibrinogen in the incubation medium, fibrinogen-specific monoclonal antibodies with an epitope in the β12−25 fragment of fibrinogen molecule that did not interact with fibrin desAB were used in the sandwich ELISA according to the standard procedure [18].

Western-blot analysis was carried out according to the standard protocol with some modifications [19]: as detecting antibodies were used monoclonal antibodies 1−5A and 1−3C, which are specific for fibrin(ogen) fragments in Aα505-610 and Bβ118−134, respectively. Anti-mouse antibodies conjugated with horseradish peroxidase were used as the conjugate.

Activated parcial thromboplastin time (APTT) was determined according to the [16].

Prothrombin time (PT) was determined according to the [16].

The action of chitin derivatives was determined in model systems:

a) whole bovine blood + chitin derivatives;

b) human blood plasma + chitin derivatives;

c) bovine and human fibrinogen + chitin derivatives.

The coagulation time was determined visually for the formation of a clot or flakes.

The statistical analysis of the experimental results were performed with Excel 2003 and Statistica 7 programmes. The article includes results lieing within error not exceeding 7% ($P < 0.07$). The data presented are typical for a series of repeated experiments (at least three in each series).

Results and Discussion

The technological use of chitin, including clinical practice, is very limited due to its complete insolubility in all types of solvents [1, 5, 13]. Because of this, the attention of the researchers is confined, first of all, to various kinds of chitin derivatives, which have full or partial solubility. We used chitosan, that is, a deacylated form of chitin, which has a solubility in aqueous solutions at pH below 8.0 [2, 15] Chitosan derivatives were also used — carboxymethylchitosan and lactate chitosan, which have higher solubility compared to chitosan by introduced functional groups [20].

Non-acylated chitin, which became soluble due to carboxylation by the carboxyl group, that is carboxymethylchitin, was used as proof that the ability to precipitate fibrinogen is not associated with the deacylated aminogroup of chitin but to the crude structure of its molecule.

It was shown that chitin and its derivatives equally caused the clot formation in blood plasma and fibrinogen solution of physiological concentration (2 mg/ml) (Table 1). At the same time, if in the blood plasma the precipitate could be characterized as a weak clot, the fibrinogen formed a typical protein precipitate without any stabilization. Thus, the cellular component of the regulation of blood coagulation is not necessary to initiate the clot formation process. The possibility of platelets activation by chitosan has been debatable for some time as well [21]. Moreover, the effective doses of the chitosan derivatives were higher for whole blood, and the duration of the its action was longer. That is, the presence of the cellular component of the regulation of the haemostasis system did not accelerate or enhance the effect of chitin derivatives on fibrinogen; it, or some other factors of whole blood, on the contrary, inhibited the chitosan effect [6, 22, 23].

The note: whole blood, blood plasma, fibrinogen solution do not form clot during experiment in normal condition. That's why the control is absent because it is without clot apriori.

Table 1. **Dependence of the time clot formation in whole blood, blood plasma and fibrinogen solution from chitin and its derivatives concentration**

Preparate name	Preparate concentration, mkg/ml	The time clot formation. s		
		Whole blood	Blood plasma	Fibrinogen solution
Chitosan	400	60±12.1	10±1.5	10±1.5
Chitosan	160	120±9.5	20±1.0	20±0.9
Chitosan	80	no clot	60±11.4	60±10.3
Chitosan	40	absence	no clot	no clot
Lactate chitosan	500	55±1.8	immediately	immediately
Lactate chitosan	100	1800±61.6	40±2.0	30±1.7
Lactate chitosan	50	no clot	120±10.1	50±2.2
Lactate chitosan	25	no clot	no clot	no clot
Carboxymethyl chitin	120	no clot	30±1.8	30±1.8
Carboxymethyl chitin	60	no clot	40±1.9	40±2.0

Note: whole blood, blood plasma, fibrinogen solution do not form clot during experiment in normal condition. That's why the control is absent because it is without clot apriori.

Absence of necessity in the platelet activation to initiate fibrinogen precipitate/clot formation due to the effect of chitin derivatives can be easily explaining the results of the action of derivatives on the blood coagulation system (Table 2). To assess the degree of influence of blood coagulation factors on the formation of a clot in the presence of chitin derivatives, coagulological tests of APTT and PT were performed. The study showed that the clot formation was no way accelerated at both the activation of the internal (APTT) and the external blood coagulation paths (PT). Moreover, in the presence of some derivatives, namely lactate chitosan and, in a less extent, carboxymethyl chitin, there was a tendency to prolongation of coagulation time. That is, these derivatives acted more like inhibitors of the process, rather than the triggers of the clot formation.

The results obtained using both whole blood and blood plasma and fibrinogen solution are almost identical. This suggests that there is no activation of the blood coagulation system under the chitosan action, and pseudoclot is formed as a result of the fibrinogen sedimentation. The elongation of the coagulation time in the APTT and PT tests under the action of chitosan derivatives can be explained precisely with this. The fibrinogen sedimentation reduces its concentration, and therefore the rate of its involvement with thrombin in the clot formation process.

It is interesting to note that the elongation of the clot formation time and the increasing of an effective dose of chitosan or its derivatives when used as a model system of whole blood is interesting. The only difference between whole blood and blood plasma is the presence of cellular components in it, primarily platelets, red blood cells and leukocytes [22, 24]. Excluding the possibility of activating the blood coagulation system, the cell component can modulate the effect of chitosan due to two factors: sorption on the membranes due to hydrophobic interaction and because of changes in the ionic medium. Unlike chitin, chitosan is not hydrophobic, so its ability to interact with membranes is unlikely. At the same time, chitin, possibly chitosan, has a very high affinity for calcium, so high that demineralization is a necessary stage of chitin purification [25–27]. Calcium is necessary to maintain the tertiary structure of fibrinogen, so, in the case of the calcium extraction by chitosan, there is a high probability of loss of fibrinogen solubility due to of the tertiary structure disorder.

To test this assumption, experiments were conducted to investigate the effect of chitosan on the formation of clusters in blood plasma and fibrinogen solution, provided that different amounts of $CaCl_2$ were introduced into the incubation solution (Table 3). It should be noted that in the case of activation of the factors of the system of blood clotting, the increase of concentration of calcium ions is a factor that accelerates the formation of a clot [28, 29]. Instead, in our case, the increase of calcium dose-dependent delayed the effect of chitosan and its derivatives. Consequently, it can be concluded that as a result of the action of chitin derivatives there is no activation of the factors of the blood coagulation system and the complete fibrin clot formation, and a fibrinogen precipitate is formed due to a disorder of its tertiary conformation due to lack of calcium.

The absense of fibrinogen to fibrin transformation was tested electrophoretic with the confirmation of native fibrinogen by Western blot method and by immunoassay with differential detection by antibodies to fibrinogen and fibrin. On an electrophoresis, in a native environment, a zone with a molecular weight corresponding to the native fibrinogen (Fig. 1) is clearly visible.

Western blot using monoclonal antibodies 1–5A to the fibrinogen/fibrin alpha-chain it is confirmed that a clear electrophoresis zone is formed precisely by a protein that contains the fibrin(ogen) alpha-chain. Using monoclonal antibodies 1–3c to fibrin it has been shown that in precipitates induced by chitin derivatives no more than 5.65 ± 0.03%

Table 2. **The chitosan and its derivatives effect on coagulation time in PT and APTT diagnostic tests**

Preparate, mkg	PT, s	APTT, s
Control	45.0±0.42	50.0±0.4
Chitosan, 100	46±0.28*	48.0±0.36**
Lactate chitosan, 100	53±0.34*	66.0±0.8**
Carboxymethyl chitin, 120	50±0.38*	56.0±1.9**

* — $P \leq 0.001$;
** — $P \leq 0.01$ compared to control.

Table 3. **The dependence of coagulation time from Ca^{2+} concentration**

Preparate, mkg	In Ca^{2+} absence, s control	20 mM Ca^{2+}, s	30 mM Ca^{2+}, s	40 mM Ca^{2+}, s
Chitosan, 600	34.0±0.36	75.2±12.1*	133±3.5**	240±11.7**
Lactate chitosan, 1000	25.0±0.28	85±10.8*	162±2.1**	>300
Carboxymethyl chitin, 1000	21.0±0.2	58±9.5*	157±4.1***	no clot

* — $P \leq 0.001$; ** — $P \leq 0.01$; *** — $P \leq 0.05$ compared to control, s — seconds

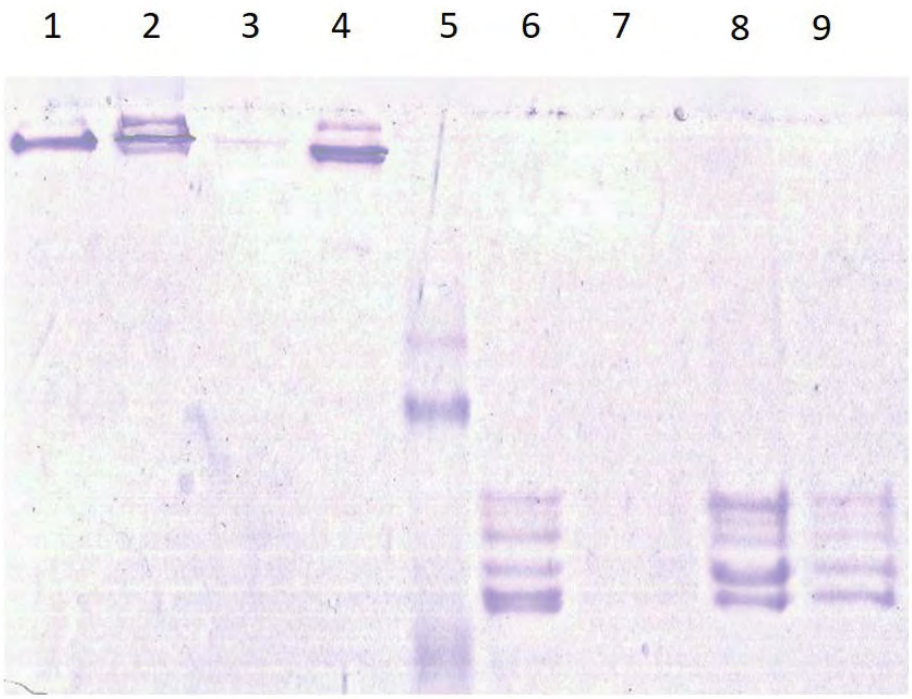

Fig. 1. **Electrophoregramme of fibrinogen samples incubated with chitin derivatives:**
at left – in absence of β-merkaptoethanol: *1* — fibrinogen; *2* — carboxymethyl chitin; *3* — chitosan; *4* — lactate chitosan; at right — in presence of β-merkaptoethanol; *6* — carboxymethyl chitin; *7* — chitosan; *8* — fibrinogen (Aα-, Bβ- and γ- chains are presented); *9* — lactate chitosan; *5* — molecular weight kit: 120, 85, 55 kDa

fibrin is present (Fig. 2). The obtained data are the same as the ones we received earlier that, in the case of non-enzyme sedimantation of fibrinogen, its precipitate may contain up to 5.0 ± 1.2% fibrin due to the action of thrombin. Trace amounts of thrombin under physiological conditions are present in blood plasma always [30]. An immuno-enzymatic assay with differential detection by antibodies to fibrinogen and fibrin confirmed that the precipitate was formed primarily by fibrinogen (93%) with a small fibrin admixture (7%).

In order to finally clear the possibility of activating the coagulation factors, an experiment was conducted on the direct determination of thrombin activity in relation to the chromogenic substrate S2238 in the case of blood plasma incubation with chitin derivatives. After more than 40 min of incubation, chitin derivatives have no effect on the activation of thrombin, that is, these compounds not promote the activation of the coagulation system, nor yet act as non-specific suppressors.

Thus, it can be confirm that chitin derivatives do not activate either the coagulation system, or the cellular component of the haemostasis system. It consists with the literature data [6, 23]. Consequently, their contact with blood plasma does not result in the transformation of fibrinogen into fibrin, and leads to the deposition of fibrinogen due to disturbance of its conformation [30, 31].

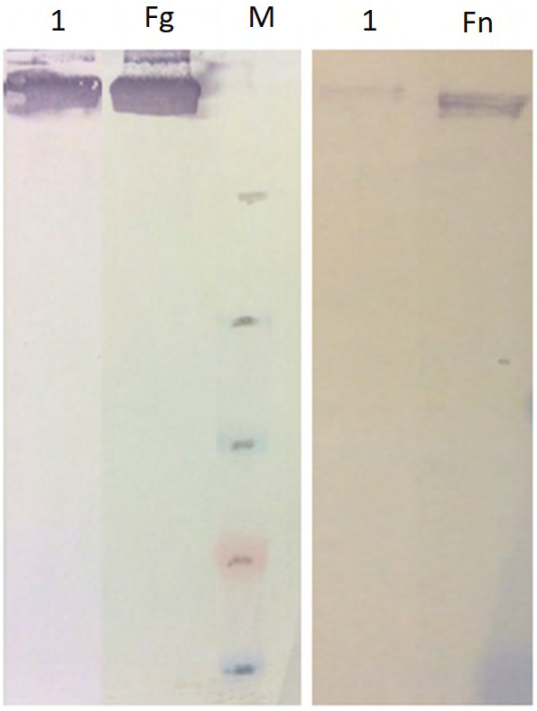

Fig. 2. **Western-blot of the fibrinogen samples incubated with carboxymethyl chitin use of monoclonal antibodies:** 1-5A (at left) and monoclonal antibodies 1-3c (at right); *M* — molecular weight kit, kDa: 250, 130, 100, 70, 55; Fg — fibrinogen, Fn — fibrin

Taking into account educed the inhibition of the effect of chitin derivatives on fibrinogen by high concentrations of calcium, it can be assumed that the disorder of fibrinogen molecule conformation is caused by the specific binding of calcium to chitin derivatives.

In view of the above, the precipitate formed under action of chitin derivatives is unphysiologic and, most importantly, it does not lead to activation of the plasminogen/plasmin system [32, 33]. As a result, such a precipitate can not be physiologic hydrolysis, so it can be remove from the wound by surgical method only. Such a feature of the chitin derivatives action makes it inappropriate for them to be used in clinical practice.

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Conflicts of Interest

The authors declare no conflict of interest.

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МЕХАНІЗМ УТВОРЕННЯ ЗГУСТКІВ У ПЛАЗМІ КРОВІ ПІД ДІЄЮ ПОХІДНИХ ХІТИНУ

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Метою роботи було з'ясування механізму утворення згустку під дією похідних хітину. Під час виконання досліджень використано біохімічні та імунологічні методи: одержання фібриногену, похідних хітозану, електрофорез у ПААГ, вестерн-блот, ELISA, коагулологічні тести, активований частково тромбопластиновий час та протромбіновий час. Отримано такі результати: похідні хітину однаковою мірою спричинюють утворення згустку в цільній крові, плазмі крові та розчині фібриногену; осад фібриногену, утворений внаслідок їхньої дії, практично не містить фібрину; хітозан не спричинює активації факторів системи зсідання крові, що підтверджується відсутністю новоутвореного фібрину; у разі додавання до розчину фібриногену хлориду кальцію останній концентраційнозалежно пригнічує ефект хітозану.

Таким чином, показано, що під дією хітозану утворюється осад фібриногену внаслідок дестабілізації його молекули через втрату кальцію. Відсутність продуктів деградації фібрину виключає можливість активації фібринолітичної системи та фізіологічну деградацію згустку. Це робить недоцільним використання кровоспинних засобів на основі хітозану в клінічній практиці.

Ключові слова: хітин та його похідні, фібриноген, система гемостазу.

МЕХАНИЗМ ОБРАЗОВАНИЯ СГУСТКОВ В ПЛАЗМЕ КРОВИ ПОД ДЕЙСТВИЕМ ПРОИЗВОДНЫХ ХИТИНА

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Целью работы было выяснение механизма образования сгустка под действием производных хитина. В работе использованы биохимические и иммунологические методы исследования: получение фибриногена, производных хитозана, электрофорез в ПААГ, вестерн-блот, ELISA, коагулологические тесты, активированное частичное тромбопластиновое время и протромбиновое время. Получены следующие данные: производные хитина в одинаковой степени вызывают образование сгустка в цельной крови, плазме крови и в растворе фибриногена; осадок, образованный в результате их действия, практически не содержит фибрин, а является, по сути, осадком фибриногена; хитозан не вызывает активацию факторов системы свертывания крови, что подтверждается отсутствием вновь образованного фибрина; при добавлении к раствору фибриногена хлорида кальция последний концентрационнозависимо подавляет эффект хитозана.

Таким образом, под действием хитозана образуется осадок фибриногена в результате дестабилизации его молекулы из-за потери кальция. Отсутствие продуктов деградации фибрина исключает возможность активации фибринолитической системы и физиологической деградации сгустка. Это делает нецелесообразным использование кровоостанавливающих средств на основе хитозана в клинической практике.

Ключевые слова: хитин и его производные, фибриноген, система гемостаза.

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