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Морфологические, гемостазиологические и гемостатические аспекты системного применения экзогенного фибрин-мономера в модели с посттравматическим кровотечением на фоне приема варфарина

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АННОТАЦИЯ

Введение. Ранее нами была установлена способность экзогенно введенного фибрин-мономера (ФМ) в низкой дозе значительно ограничивать посттравматическую кровопотерю на экспериментальной модели варфариновой коагулопатии «*in vivo*». При этом морфологические особенности прираневого фибринообразования не были рассмотрены.

Цель. Сравнить морфологические, гемостазиологические и гемостатические данные по итогам системного применения экзогенного ФМ для интерпретации его эффектов в модели с посттравматическим кровотечением на фоне приема варфарина.

Материалы и методы. В работе использовались кролики-самцы породы Шиншилла. Проведен сравнительный анализ гемостазиологических эффектов и морфологической картины прираневой поверхности печени после дозированной травмы при предварительном системном введении ФМ (0,25 мг/кг внутривенно) или концентрата факторов протромбинового комплекса (40 МЕ/кг внутривенно) на фоне приема животными варфарина (0,4–0,5 мг/кг/сут *per os* в течение 2-х недель).

Результаты. Введение извне ФМ у варфаринизированных животных в условиях дозированной экспериментальной травмы печени способствовало гемостатическому эффекту, сравнимому с действием концентрата факторов протромбинового комплекса. Оба гемостатических препарата приводили к интенсивному фибриноообразованию, способствующему уменьшению посттравматической кровопотери. В случае применения ФМ отмечалось локализованное в раневой поверхности увеличение толщины тромботических отложений и фибриновых нитей в сравнении с плацебо в 4,0 раза и в 1,6 раза соответственно ($p < 0,000001$). В этот процесс активно вовлекались тромбоциты, что приводило к снижению их количества в просвете прираневых сосудов в 1,7 раза ($p < 0,0002$). Не было выявлено какого-либо действия со стороны ФМ на системные гемостатические реакции в венозной крови в отличие от концентрата факторов протромбинового комплекса.

Заключение. Введенный извне ФМ способен оказывать локальное гемостатическое действие в условиях дозированной экспериментальной травмы и коагулопатии, вызванной приемом варфарина. Гемостатическое действие было опосредовано интенсивным тромбообразованием на раневой поверхности печени с активным вовлечением тромбоцитов в процесс. Особенности продемонстрированных эффектов ФМ могут быть опосредованными через пока еще не установленные механизмы действия ФМ, что определяет необходимость в продолжении исследований в данном направлении.

Ключевые слова: *фибрин-мономер; варфарин; концентрат факторов протромбинового комплекса; травма печени; остановка кровотечения; образование фибрина*

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Morphologic, Hemostasiologic and Hemostatic Aspects of Systemic Application of Exogenous Fibrin Monomer in Model of Posttraumatic Bleeding with Underlying Intake of Warfarin

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ABSTRACT

INTRODUCTION: Earlier, an ability of exogenous fibrin monomer (FM) introduced at low doses to considerably limit posttraumatic blood loss was established by us on an experimental model of warfarin coagulopathy *in vivo*. However, the morphologic peculiarities of fibrin formation in the wound area were not considered.

AIM: To compare morphologic, hemostasiologic and hemostatic data based on the results of systemic application of exogenous FM to interpret their effects in the model of posttraumatic bleeding with the underlying intake of warfarin.

MATERIALS AND METHODS: In the work, Chinchilla male rabbits were used. A comparative analysis of hemostasiologic effects and of morphologic picture of the surface of the liver in the wound area was conducted after a dosed trauma, with a preliminary systemic introduction of FM (0.25 mg/kg intravenously) or a concentrate of prothrombin complex factors (40 IU/kg intravenously) with the underlying intake of warfarin by animals (0.4–0.5 mg/kg/day per os for 2 weeks).

RESULTS: Introduction of FM in warfarinised animals in the conditions of a dosed experimental liver injury promoted a hemostatic effect comparable with that of a concentrate of prothrombin complex factors. Both hemostatic drugs led to intense fibrin formation that reduced posttraumatic blood loss. The use of FM was associated with increase in the thickness of thrombotic deposits and fibrin fibers in the wound surface in comparison with placebo by 4.0 and 1.6 times, respectively ($p < 0.000001$). This process actively involved platelets, which led to 1.7 times reduction of their quantity in the lumen of the blood vessels in the wound vicinity ($p < 0.0002$). No effect of FM on systemic hemostatic reactions in venous blood was found, in contrast to concentrate of prothrombin complex factors.

CONCLUSION: Exogenous FM can produce a local hemostatic effect in the conditions of dosed experimental trauma and coagulopathy induced by warfarin intake. The hemostatic effect was mediated by intense thrombosis on the wound surface with the active recruitment of platelets in the process. The peculiarities of the demonstrated effects of FM may be mediated though the mechanisms of its action that have not yet been identified, which necessitates continuation of the research in this direction.

Keywords: *fibrin monomer; warfarin; concentrate of prothrombin complex factors; hepatic injury; bleeding arrest; fibrin formation*

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ECO•VECTOR

LIST OF ABBREVIATIONS

A10 — clot amplitude in 10 min
CFT — clot formation time
CPCF — concentrate of prothrombin complex factors
CT — coagulation time
ETP — endogenous thrombin potential
F — fibrin
FM — fibrin monomer
INR — international normalized ratio
i/v — intravenously
MCF — maximum clot firmness
m.f. — microscopic field
MSB — martius scarlet blue
n.r. — not recorded
Plt — platelets
TBV — total blood volume
ttPeak — time to peak
$V_{thrombin}$ — thrombin generation velocity index

INTRODUCTION

Researchers in all countries of the world are studying hemostasis as an integral, multicomponent and self-regulated system. The works published in recent years show that the regulatory mechanisms of this system have not yet been fully discovered. This is evidenced by the statements of the modern theory of cellular model of hemocoagulation [1–3], as well as by new ideas about the peculiarities of spatial thrombinogenesis and fibrinogenesis in *in vitro* conditions [4]. These circumstances create prerequisites for continuation of investigation of the mechanisms of hemostatic reactions *in vivo*, which may be helpful in further development of new effective and safe hemostatic drugs.

For many years, in the classic cascade scheme of fibrin formation by A. A. Schmidt and P. O. Morawitz, a molecule of fibrin monomer (FM) as a product of proteolytic action of thrombin on fibrinogen, was considered only as *a substrate for formation of polymer fibrin fibers* [5, 6]. Analyzing the data of modern studies and classic ideas about the mechanisms of hemostatic reactions, we put forward a hypothesis that suggested the existence of a regulatory effect of FM on the processes of intravascular thrombosis. This assumption was rather convincingly confirmed on models in several *in vivo* experiments performed by our research team. Thus, it was established that intravenous administration of this drug at a dose of 0.25 mg/kg, without affecting hemostasiologic parameters, leads to minimization of posttraumatic blood loss (in dosed liver injury) in different models of hypocoagulation with the intake of antithrombotic drugs such as heparin [7], dabigatran etexilate [8], or in suppression of platelet function [9]. To note, the used dose of exogenous FM after dilution in the systemic bloodstream, becomes comparable with its physiological content in blood plasma in healthy individuals and does not exceed 7.8 μg/ml [10]. Therefore, we are not inclined to assign the hemostatic effects of FM at this dose to non-localized increase in systemic hemostatic activity.

Nevertheless, investigation of the morphologic picture of the wound surface after administration of FM to animals receiving heparin or antiplatelet agents, showed enhanced fibrin formation that predetermines reduction of blood loss [7, 9].

The hemostatic properties of FM in warfarin-induced coagulopathy were analyzed by us earlier. The data obtained showed that in terms of the intensity of blood loss reduction, FM is not inferior to the comparison drug — concentrate of prothrombin complex factors (CPCF). However, morphologic aspects of fibrin formation in the wound area were not presented on this model.

The **aim** of this study to compare morphologic, hemostasiologic and hemostatic data of systemic application of exogenous fibrin with underlying intake of warfarin.

MATERIALS AND METHODS

On the base of Altai State Medical University, a study has been conducted on male Chinchilla rabbits (n = 64) of 3.0–4.5 kg mass. The work was approved by the Local Ethics Committee of Altai State Medical University (Protocol No. 12 of 2015, November 12). The experiments on animals were conducted according to the requirements of 2010/63/EC Directive of the European Parliament and of the Council of the European Union on the protection of animals used for scientific purposes (of 2010, September 22), the European Convention for the protection of vertebrate animals used for experiments or for other scientific purposes (of 1986, March 18), as well as Declaration of Helsinki and Rules for performing works using experimental animals.

Using the block randomization method, 4 groups of animals were formed.

Group 1 (n = 21): the animals were injected 0.5 ml of placebo solution into the marginal vein of the ear (urea solution (3.75 M) at a concentration comparable with that in FM drug)). After one hour, midline laparotomy was performed under general anesthesia, and a standard

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liver injury was inflicted by the established method to evaluate the volume and rate of blood loss, as well as fibrin formation in the wound [12]. For anesthesia, animals were injected Telazol® at a dose of 10 mg/kg (Zoetis, Russia).

Group 2 (n = 13), ***group 3 (n = 14)*** and ***group 4 (n = 16)***: animals were previously introduced aqueous solution of warfarin per os at a dose of 0.4–0.5 mg/kg (Nycomed, Denmark). The animals in which the value of international normalized ratio (INR) reached ≥ 2.0, were used in the experiment. Further, these animals were intravenously injected placebo solution in the amount of 0.5 ml, CPCF solution (Prothrombin 600®, Baxter, Austria) at a dose of 40 IU/kg or FM solution (Tekhnologia-Standart, Russia; RF Patent No. 2522237 of 2014, July 10) at a dose of 0.25 mg/kg. In 60 minutes after i/v introduction of placebo or systemic hemostatic agent, a standard liver injury was performed [12] under general anesthesia with Telazol® (Zoetis, Russia).

The system of hemostasis was evaluated in venous blood obtained from the marginal vein of the ear in phlebotomy. Blood was taken before i/v introduction of the preparations (hemostatic agent or placebo), and also immediately before anesthesia prior to modeling of wound bleeding. To count the number of platelets, the venous blood taken in this way, was stabilized by placement into test tubes with potassium salts of ethylene diamine tetracidic acid (AQUISEL® K3E/EDTA 3K, Aquisel S. L., Spain). In the obtained samples, *platelets were counted* using Drew-3 automatic hematology analyzer (Drew Scientific Inc., Great Britain-USA). Other parameters of hemostasis were studied with 0.11 M (3.8%) sodium citrate solution (9:1 ratio) used as blood stabilizer. From these samples, platelet-poor plasma was obtained by standard methods, in which *INR and platelet concentration were determined by Klaus method* on Thrombostat 2 semi-automatic coagulometer (Behnk Electronik, Germany) with an appropriate reagent kit (Tekhnologia-Standart, Russia). Besides, *D-dimer level* was evaluated on an analyzer-reflectometer using NycoCard® D-Dimer test system (Axis-Shield PoC AS, Norway). Stabilized whole citrated blood was also used for *thromboelastometry* on ROTEM® Gamma thromboelastometer (Pentapharm GmbH, Germany) in *Natem* mode with Startem reagent.

Standard parameters of thromboelastogram were evaluated:

- coagulation time (CT);
- clot formation time (CFT);
- clot amplitude (α angle);
- maximum clot firmness (MCF);
- clot amplitude in 10 minutes (A10).

In platelet-poor blood plasma, *thrombin generation was evaluated by method of calibrated automated thrombography* according to H. C. Hemker (2003). The study was conducted on Fluoroskan Ascent plate fluorimeter with Thrombinoscope® 3.0.0.26 software (ThermoFisher SCIENTIFIC, Finland) with Thrombinoscope® reagents (*PPP-Reagent, Thrombin Calibrator, FluCa-Kit*; the Netherlands).

The following parameters were evaluated:

- time of initiation of thrombin formation (lagtime);
- endogenous thrombin potential (ETP);
- peak thrombin concentration;
- time to peak thrombin concentration (time to peak, ttPeak);
- thrombin formation velocity ($V_{thrombin}$) [13].

For ***histologic examination***, the liver tissue was taken from the animals including a part of wound and a fragment of intact surface with subsequent fixation in 10% neutral formalin solution by Lilly method. The biomaterial was obtained immediately after spontaneous stoppage of bleeding. In cases of animal death with continuing bleeding, the biomaterial was taken immediately after stoppage of cardiopulmonary activity. The tissue was processed with isopropyl alcohol in carousel automat (TISSUE-TEK VI PTM6 model, Sakkura, Japan) and was embedded in paraffin on mod. TISSUE-TEK TEC 5 station (Sakkura, Japan).

Histologic sections of 4–5 μm thickness were obtained on semi-automatic rotor microtome (mod. Accu-Cut SRM, Sakkura, Japan) with subsequent staining with hematoxylin and eosin in a device for automated staining of micropreparations (mod. TISSUE-TEK Prisma, Sakkura, Japan). Further, they were transferred under the film in the device for automatic processing of micropreparations (mod. TISSUE-TEK Film, Sakkura, Japan). The microscopic structure of fibrin deposits in the micropreparations was studied after preliminary staining of sections by MSB method according to D. D. Zerbino and L. L. Lukasevich [14]. The fibrin age was identified using reagents (BVS, Russia).

Platelets were counted in large vessels of venous or arterial type in stained micropreparations in five microscopic fields in immersion microscope (magnification × 1000). After that, the mean number of these cells was calculated. Microphotographs were obtained in Leica DM 750 E200 microscope with Leica EC3 digital videocamera (Leica Microsystems CMS GmbH, Germany). The obtained images were analyzed using Image Tool. 3.0 software.

The animals were subjected to *euthanasia* after stoppage of bleeding from the wound by introduction of an anesthetic at a lethal dose (3–4 times exceeding the therapeutic one) or at the moment of arrest of cardiopulmonary activity irrespective of the continuation of bleeding.

Statistical analysis of the results was performed on the basis of MedCalc Version 17.9.7 software (license number: BU556-P12YT-BBS55-YAH5M-UBE51).

The significance of differences between the parameters was evaluated using Mann–Whitney test or Wilcoxon W-test. The differences were considered statistically significant at $p \leq 0.05$. Distribution of values is presented as median (Me) with indication of 25th and 75th percentiles (Q25–Q75).

RESULTS

In a previously published paper, we demonstrated structural alterations in histological micropreparations of hepatic tissue in the trauma area in intact animals with introduction of different doses of exogenous FM [15]. In this article we partially reproduced these data as a starting point for analysis of the morphologic picture in animals receiving anticoagulant therapy including hemostatic agents of systemic effect.

Morphologic Data

In the placebo group 1, thrombotic deposits in the form of thin, smooth shiny pinkish masses about 66 μm thickness (Figure 1A) were detected in the area of the surgical wound (Table 1). These formations consisted mainly of fibrin in the form of thin, rarely anastomosing fibers (Figure 1B). Fibrin fibers were mainly oriented along the wound surface. Among them, unchanged erythrocytes were found. According to the criteria stated by D. D. Zerbina and L. L. Lukasevich in their work [14], thrombotic masses on the wound surface in animals of group 1, as well as in other groups 2–4 may be referred to mixed type thrombi (*fibrin-erythrocyte*). It should also be noted that there were about 73 platelets in the microscopic field in the lumen of large vessels near the wound (Figure 1C).

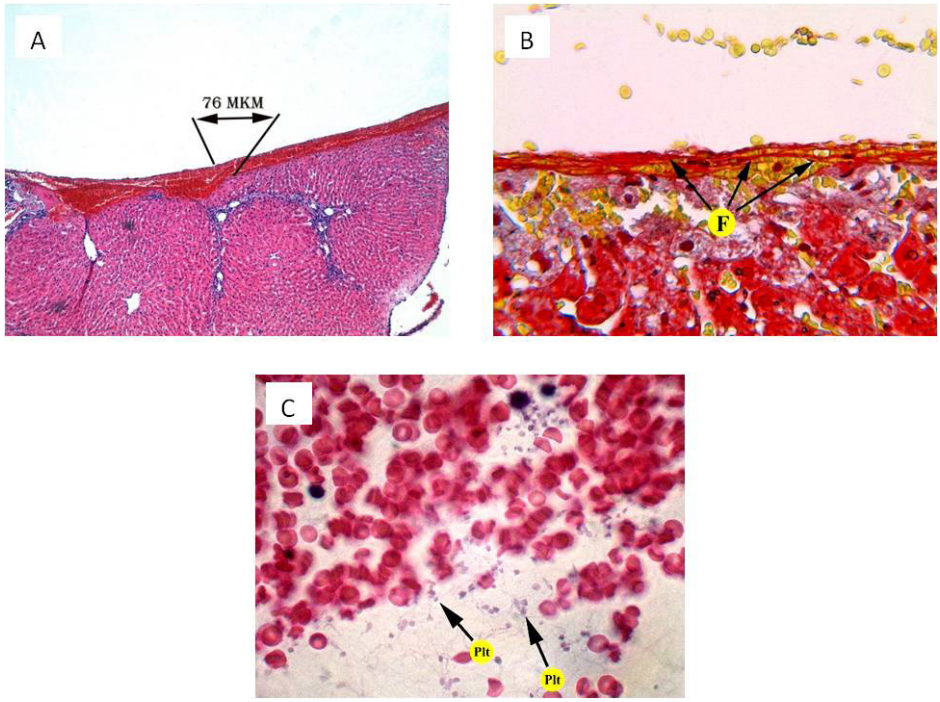

Fig. 1. Morphologic changes in the liver wound area after spontaneous stoppage of bleeding on an example of a rabbit from the placebo group: A — thrombotic masses, hematoxylin and eosin stain, × 100 magnification; B — fibrin fibers (arrows) in thrombotic masses, MSB stain for fibrin, × 400 magnification; C — lumen of large vessels in the wound area containing platelets (arrows), hematoxylin and eosin stain, × 1000 magnification.
Notes: MSB — martius scarlet blue, F — fibrin, Plt –platelets.

In warfarin-treated animals after introduction of placebo (group 2), pink, smooth thrombotic deposits of small thickness (Me = 25 μm) were determined on the wound surface (Figure 2A, Table 1) that were 2.6 times less expressed in comparison with group 1. They consisted of relatively thin, pink fibrin fibers (Figure 2B) oriented mainly along the wound surface. Anastomoses between them were scarce, like in group 1. Fibrin fibers were 3.1 times thicker in comparison with group 1. Besides, there were inclusions of small numbers of unchanged erythrocytes in thrombotic masses. With this, the number of platelets in the lumen of large vessels in the wound area (Figure 2C) did not differ from group 1.

Table 1. Morphometric Characteristics of Histologic Micropreparations of Liver Injury

Parameters	Group 1 (after introduction of placebo)	Group 2 (after introduction of warfarin and placebo)	Group 3 (after introduction of warfarin and CPCF)	Group 4 (after introduction of warfarin and FM)
n	21	13	14	16
Thickness of thrombotic masses, μm	66.2 [62.7–83.5]	25.8 [24.1–34.3] $p_{1-2} < 0.000001$; $\Delta \times 2.6$	79.3 [72.8–84.9] $p_{1-3} = 0.040$; $\Delta \times 1.2$; $p_{2-3} < 0.000001$; $\Delta \times 3.1$	102.0 [81.8–115.1] $p_{1-4} = 0.002$; $\Delta \times 1.5$; $p_{2-4} < 0.000001$; $\Delta \times 4.0$; $p_{3-4} = 0.012$; $\Delta \times 1.3$
Thickness of fibrin fibers, μm	0.83 [0.72–0.93]	2.59 [1.96–2.90] $p_{1-2} = 0.005$; $\Delta \times 3.1$	5.57 [3.91–6.99] $p_{1-3} = 0.005$; $\Delta \times 6.7$; $p_{2-3} = 0.00001$; $\Delta \times 2.2$	4.03 [3.56–4.61] $p_{1-4} = 0.005$; $\Delta \times 4.9$; $p_{2-4} = 0.00001$; $\Delta \times 1.6$; $p_{3-4} = 0.151$
Number of platelet, pc./m.f.	73.5 [61.0–90.8]	81.0 [62.0–96.0] $p_{1-2} = 0.836$	56.5 [47.8–69.5] $p_{1-3} = 0.026$; $\Delta \times 1.3$; $p_{2-3} = 0.005$; $\Delta \times 1.4$	48.5 [41.3–57.5] $p_{1-4} = 0.002$; $\Delta \times 1.5$; $p_{2-4} = 0.0002$; $\Delta \times 1.7$; $p_{3-4} = 0.199$

Notes: CPCF — concentrate of prothrombin complex factors, m.f. — microscopic field, FM – fibrin monomer, Δ — difference in compared parameters

In case of using CPCF instead of placebo (group 3), the thickness of thrombotic deposits was 1.2 times that (Figure 3A) in group 1 and 3.1 that in group 2 (Table 1) and made about 79 μm. Thrombotic masses were of brownish shade and had a knobby surface. They contained a large number of predominantly lysed erythrocytes and 'thick' strands of fibrin (shown by arrows in Figure 3B). There structures, as a rule, ran parallel to the wound surface of the liver, contained thickenings along the route and anastomosed with each other. To note, the thickness of fibrin fibers in group 3 was maximal and was 6.6 and 2.2 times the thickness in group 1 and 2, respectively. Along with increase in thickness of thrombotic masses and of fibrin fibers in the lumen of large vessels, a decrease in the number of platelets was noted (shown by arrows in Figure 3C) 1.3 times in comparison with group 1 and 1.4 times in comparison with group 2.

In the experimental group of animals that received FM as a hemostatic agent (group 4), the wound surface was covered with thick (about 102 microns) brownish thrombotic masses with an uneven (knobby) surface (Table 1). Thrombotic deposits in this group 4.0 times exceeded the thickness of deposits in group 2 and 1.3 three times — in group 3. Thrombotic masses contained a large number of lysed and unchanged erythrocytes, as well as thickened fibrin fibers that ran in different directions forming numerous anastomoses (Figure 4B). The thickness of fibrin fibers in this group exceeded this parameter in groups 1 and 2, by 4.9 and 1.6 times, respectively. The preparations of this group of animals had the smallest number of platelets in large vessels in the wound area of all comparison groups (Figure 4B) that amounted to about 48 cells in the microscopic field (m.f.).

Peculiarities of Changes in System of Hemostasis in Experimental Coagulopathy

As it was shown by us earlier [11], the volume of blood loss (% of the total blood volume, TBV) in warfarin-treated animals after introduction of FM and CPCF was 9.1 and 6.7 times less as compared to the warfarin plus placebo group. Similar changes were seen in the rate of blood loss (in mg/s). This parameter was 3.7 times below the control values in FM group, and 5 times below the control in CPCF group.

Differences in the morphologic structure of the wound surface of the liver in animals of the experimental groups to a certain extent correlated with the volume

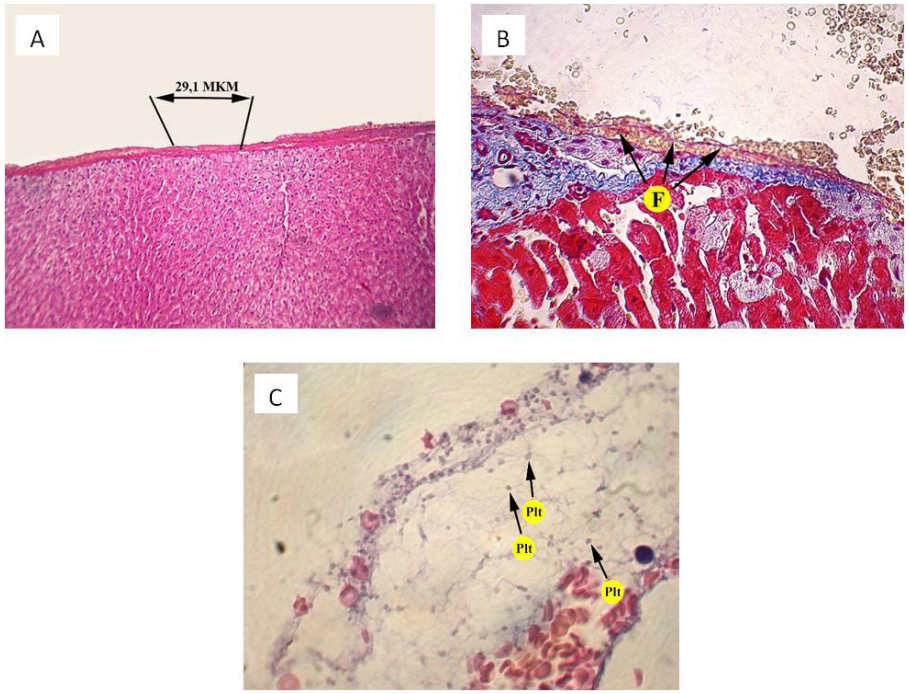

Fig. 2. Morphologic changes in the liver wound area after spontaneous stoppage of bleeding on an example of a rabbit from warfarin plus placebo group: A — thrombotic masses, hematoxylin and eosin stain, × 100 magnification; B — fibrin fibers (arrows) in thrombotic masses, MSB stain for fibrin, × 400 magnification; C — lumen of large vessels in the wound area containing platelets (arrows), hematoxylin and eosin stain, × 1000 magnification.
Notes: MSB — martius scarlet blue, F — fibrin, Plt — platelets.

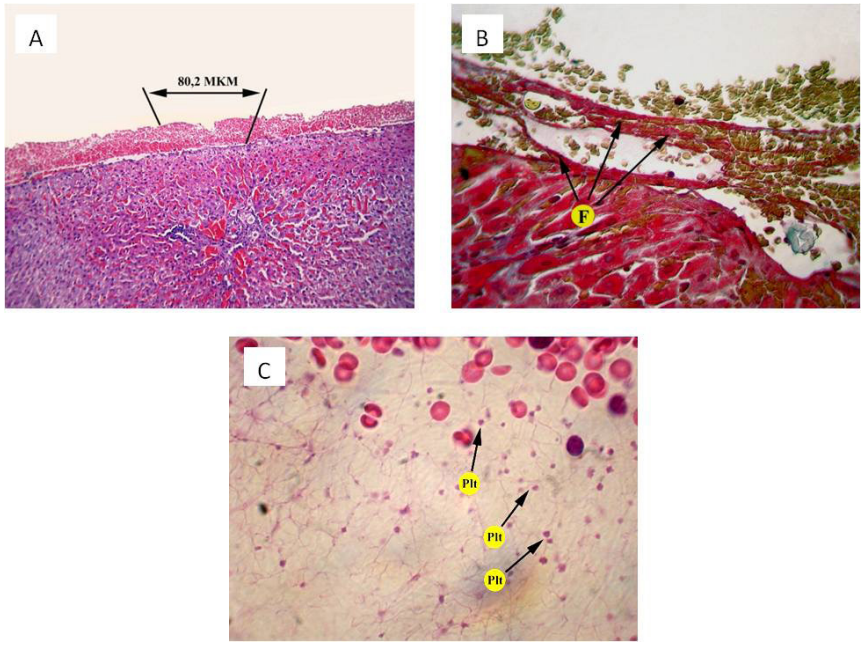

Fig. 3. Morphologic changes in the liver wound area after spontaneous stoppage of bleeding on an example of a rabbit from warfarin plus CPCF group: A — thrombotic masses, hematoxylin and eosin stain, × 100 magnification; B — fibrin fibers (arrows) in thrombotic masses, MSB stain for fibrin, × 400 magnification; C — lumen of large vessels in the wound area containing platelets (arrows), hematoxylin and eosin stain, × 1000 magnification.
Notes: MSB — martius scarlet blue, F — fibrin, Plt — platelets.

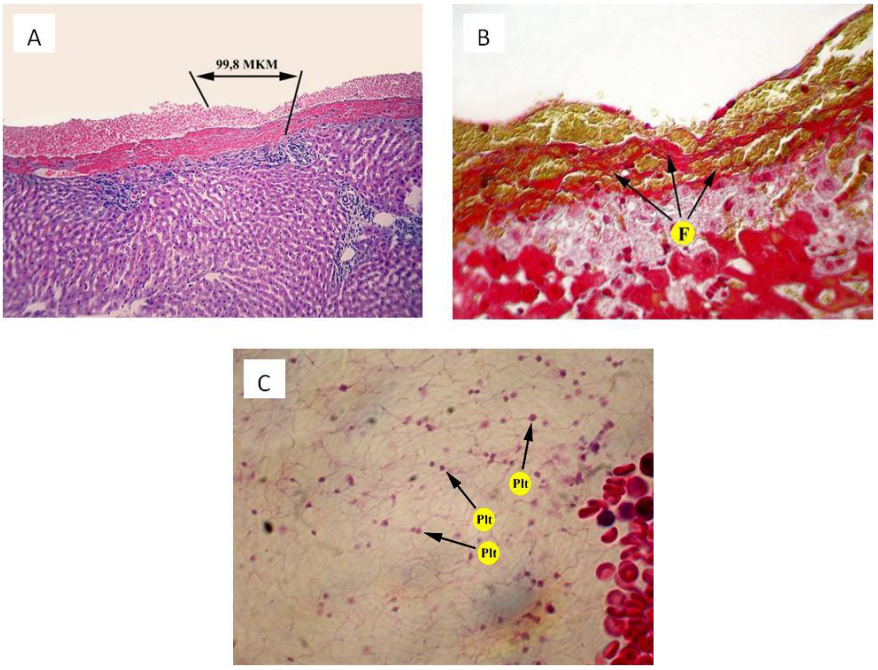

Fig. 4. Morphologic changes in the liver wound area after spontaneous stoppage of bleeding on an example of a rabbit from warfarin plus FM group: A — thrombotic masses, hematoxylin and eosin stain, × 100 magnification; B — fibrin fibers (arrows) in thrombotic masses, MSB stain for fibrin, × 400 magnification; C — lumen of large vessels in the wound area containing platelets (arrows), hematoxylin and eosin stain, × 1000 magnification.
Notes: MSB — martius scarlet blue, F — fibrin, Plt — platelets.

and rate of blood loss, as well as with hemostasiologic changes in whole venous blood and platelet-poor plasma, which is demonstrated in Table 2. It is quite obvious that the use of warfarin in groups 2–4 before the experiment was associated with the development of warfarin-related coagulopathy in the form of hypocoagulation evidenced by INR (2.0–2.5 times increase), coagulation time (CT) in thromboelastometry (2.4–3.5 times prolongation), with the impossibility to record other parameters of the method in most blood samples (in 54%–61% of cases). Along with this, there was an expected decrease in the intensity of thrombin generation in blood plasma shown by Lagtime, ETP, Peak thrombin, ttPeak and Vthrombin.

Introduction of CPCF in warfarinised animals of group No.3 as an antidote to warfarin led to correction of the coagulation shift with reversal of INR to the normal level (group No.1) and with overcompensated increase in density characteristics of the clot in thromboelastometry (Table 2). In particular, there was 1.2 times increase in MCF and 1.5 increase in A10 compared to the parameters obtained in group No.1. It is important to note the excessive enhancement of thrombin generation indicated by the leading parameters of this test.

The use of exogenous FM in animals of group 4 was not accompanied by any systemic correction of hypocoagulation induced by anticoagulant therapy (in venous blood). This was confirmed by 2.2 times increase in INR values (in median) of the respective parameter determined in the animals of group 1 (Table 2) and was associated with prolongation of chronometric parameters of blood coagulation (CT and CFT) together with the reduction of density characteristics of the clot (α angle, MCF and A10) and low intensity of thrombin generation.

DISCUSSION

The described results of the study on the model of warfarin-induced coagulopathy demonstrated fatal posttraumatic blood loss (in % of calculated TBV) and death of 75% in warfarinised animals [11]. As it is known, administration of warfarin per os results in inhibition of enzymes required for the synthesis of vitamin K-dependent blood coagulation factors (II, VII, IX, X) in hepatocytes thus leading to hypocoagulation. This reduction of blood coagulation was proved with taking into account the data of INR (in prothrombin test), of thromboelastometry and calibrated thrombography test. Here, use of FM together with the known hemostatic drug — CPCF — *was accompanied by significant reduction of parameters of posttraumatic blood loss*.

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Table 2. Changes in System of Hemostasis, Parameters of Automated Thrombography and Rotation Thromboelastometry

Parameters	Group 1		Group 2		Group 3		Group 4	
	before introduction of placebo	after introduction of placebo	before introduction of placebo with intake of warfarin	after introduction of placebo with intake of warfarin	before introduction of CPCF with intake of warfarin	after introduction of CPCF with intake of warfarin	before introduction of FM with intake of warfarin	after introduction of FM with intake of warfarin
	1a	1b	2a	2b	3a	3b	4a	4b
n	21		13		14		16	
Platelet count, × 10^9/l	477.5 [405.8–621.5]	480.5 [412.3–555.0] $p_{1a-1b} = 0.151$	555.0 [471.0–591.0]	512.0 [474.0–700.0] $p_{2a-2b} = 0.382$	425.0 [392.8–531.3]	399.0 [334.0–454.5] $p_{3a-3b} = 0.049$ Δ-6.1%	509.0 [417.8–578.0]	479.5 [408.3–551.5] $p_{4a-4b} = 0.328$
INR	1.1 [0.6–1.6]	0.9 [0.6–1.7] $p_{1a-1b} = 0.683$	2.4 [2.0–4.0]	2.5 [2.2–4.6] $p_{2a-2b} = 0.650$	2.1 [1.7–6.2]	1.1 [1.0–1.2] $p_{3a-3b} = 0.002$ Δ-47.6%	2.0 [1.6–3.6]	2.0 [1.5–2.9] $p_{4a-4b} = 0.063$
Fibrinogen g/l	3.3 [2.8–4.4]	3.7 [2.8–4.5] $p_{1a-1b} = 0.811$	2.8 [2.6–4.3]	3.0 [2.6–4.4] $p_{2a-2b} = 0.814$	3.3 [2.8–4.1]	2.9 [2.5–3.6] $p_{3a-3b} = 0.260$	3.1 [2.7–3.5]	3.0 [2.5–3.3] $p_{4a-4b} = 0.065$
D-dimer, ng/ml	100.0 [100.0–100.0]	100.0 [100.0–200.0] $p_{1a-1b} = 0.201$	150.0 [100.0–200.0]	150.0 [100.0–200.0] $p_{2a-2b} = 0.351$	100.0 [100.0–100.0]	100.0 [100.0–200.0] $p_{3a-3b} = 0.180$	200.0 [100.0–250.0]	200.0 [150.0–400.0] $p_{4a-4b} = 0.075$
Automated Calibrated Thrombography (thrombogram)								
Lagtime, min	2.2 [2.0–2.7]	2.0 [1.8-2.7] $p_{1a-1b} = 0.068$	3.5 [2.7–4.5] n.r. in 2 cases of 9	4.4 [3.4–5.6] n.r. in 3 cases of 9 $p_{2a-2b} = 0.592$	5.0 [4.3–5.3] n.r. in 6 cases of 9	1.7 [1.5–2.0] conventionally Δ -2.9 times	4.5 [4.5–5.3] n.r. in 3 cases of 9	6.0 [5.9–6.3] n.r. in 4 cases of 9 $p_{4a-4b} = 0.593$
ETP, nmol × min	373.9 [338.7–500.4]	484.8 [360.6–622.5] $p_{1a-1b} = 0.224$	150.2 [92.3–183.9] n.r. in 2 cases of 9	103.0 [60.9–158.8] нn.r. in 3 cases of 9 $p_{2a-2b} = 0.109$	97.8 [68.2–104.9] n.r. in 6 cases of 9	582.0 [444.9–806.4] conventionally Δ +6.0 times	131.7 [81.3–145.2] n.r. in 3 cases of 9	149.3 [111.3–189.6] нn.r. in 4 cases of 9 $p_{4a-4b} = 0.514$
Peak thrombin, nmol/l	76.2 [40.7–90.9]	81.7 [34.3–138.8] $p_{1a-1b} = 0.128$	28.2 [18.9–56.2] n.r. in 2 cases of 9	12.5 [7.5–21.8] n.r. in 3 cases of 9 $p_{2a-2b} = 0.041$ Δ -2.3 times	10.9 [7.3–14.8] n.r. in 6 cases of 9	65.4 [41.3–74.5] conventionally Δ +6.0 times	10.5 [10.3–13.6] n.r. in 3 cases of 9	13.3 [10.9–21.9] n.r. in 4 cases of 9 $p_{4a-4b} = 0.285$
ttPeak, min	5.8 [5.0–7.3]	5.4 [4.6–6.3] $p_{1a-1b} = 0.143$	6.5 [4.7–7.2] n.r. in 2 cases of 9	9.2 [8.3–10.5] n.r. in 3 cases of 9 $p_{2a-2b} = 0.108$	9.5 [7.9–9.9] n.r. in 6 cases of 9	9.5 [8.8–9.6] $p_{16-3b} = 0.0006$ Δ +1.8 times	10.5 [10.2–11.0] n.r. in 3 cases of 9	10.8 [10.3–11.1] n.r. in 4 cases of 9 $p_{4a-4b} = 0.922$
$V_{thrombin}$, nmol/min	25.3 [9.2–29.1]	26.8 [7.8–62.2] $p_{1a-1b} = 0.102$	9.4 [7.1–25.9] n.r. in 2 cases of 9	3.4 [2.0–6.5] n.r. in 3 cases of 9 $p_{2a-2b} = 0.085$	2.4 [1.6–4.7] n.r. in 6 cases of 9	7.8 [6.4–11.8] conventionally Δ +3.3 times $p_{16-3b} = 0.050$ Δ -3.4 times	2.3 [1.6–3.0] n.r. in 3 cases of 9	2.8 [2.3–5.2] n.r. in 4 cases of 9 $p_{4a-4b} = 0.592$

Continuation of Table 2

Parameters	Group 1		Group 2		Group 3		Group 4	
	before introduction of placebo	after introduction of placebo	before introduction of placebo with intake of warfarin	after introduction of placebo with intake of warfarin	before introduction of CPCF with intake of warfarin	after introduction of CPCF with intake of warfarin	before introduction of FM with intake of warfarin	after introduction of FM with intake of warfarin
	1a	1b	2a	2b	3a	3b	4a	4b
Rotation Thromboelastometry								
CT, sec	605.5 [453.8–801.5]	628.0 [479.0–856.0] $p_{1a-1b} = 0.821$	2122.5 [1328.3–2464.8]	2095.0 [1052.0–2398.0] $p_{2a-2b} = 0.530$	1573.5 [948.3–2394.0]	494.0 [355.0–626.0] $p_{3a-3b} = 0.002$ Δ -3,2 times	1459.0 [783.5–2198.8]	1559.5 [734.0–1918.8] $p_{4a-4b} = 0.221$
α angle, °	57.0 [46.5–62.0]	55,0 [49,0–65,0] $p_{1a-1b} = 0,207$	46.0 [40.8–49.0] n.r. in 9 cases of 13	46.5 [33.0–57.8] n.r. in 7 cases of 13	48.0 [39.5–52.0] n.r. in 8 cases of 14	68.0 [59.0–71.0] $p_{3a-3b} = 0.151$	39.5 [30.3–60.8] n.r. in 6 cases of 16	37.0 [32.8–55.3] n.r. in 4 cases of 16 $p_{4a-4b} = 0.767$
CFT, sec	182.5 [148.8–269.3]	206,0 [146,0–254,0] $p_{1a-16} = 0,288b$	354.0 [305.0–414.0] n.r. in 10 cases of 13	320.0 [180.0–437.0] n.r. in 8 cases of 13	356.0 [307.5–794.5] n.r. in 8 cases of 14	166.0 [110.0–181.0] $p_{3a-3b} = 0.028$ Δ -2.2 times	452.5 [218.5–522.5] n.r. in 8 cases of 16	367.0 [187.0–404.8] n.r. in 6 cases of 16 $p_{4a-4b} = 0.735$
MCF, mm	59.5 [56.0–64.3]	58.0 [54.0–64.0] $p_{1a-1b} = 0.956$	28.0 [24.0–38.0] n.r. in 8 cases of 13	31.0 [27.5–43.5] n.r. in 7 cases of 13	22.5 [9.0–49.5] n.r. in 4 cases of 14	70.0 [67.0–76.0] $p_{3a-3b} = 0.008$ Δ +3.1 times $p_{16-3b} = 0.005$ Δ +1.2 times	32.5 [15.8–50.5] n.r. in 6 cases of 16	44.0 [32.0–49.5] n.r. in 4 cases of 16 $p_{4a-4b} = 0.139$
A10, mm	44.0 [40.8–52.5]	43.0 [39.0–50.0] $p_{1a-1b} = 0.422$	23.5 [20.0–28.3] n.r. in 9 cases of 13	31.0 [26.0–46.0] n.r. in 8 cases of 13	8.5 [4.0–34.5] n.r. in 4 cases of 14	64.0 [55.0–68.0] $p_{3a-3b} = 0.007$ Δ +7.5 times $p_{16-3b} = 0.006$ Δ +1.5 times	24.5 [18.8–38.0] n.r. in 6 cases of 16	32.0 [27.0–41.0] n.r. in 5 cases of 16 $p_{4a-4b} = 0.260$

Notes: CPCF — concentrate of prothrombin complex factors, n.r. — not recorded, FM — fibrin monomer, Δ — difference in compared parameters

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In the present study, the wound surface in warfarinised animals was morphologically presented as insignificant thrombotic deposits with relatively thin fibrin fibers and corresponded to the expected increase in blood loss and death of animals due to insufficient effectiveness of plasma mechanisms of the system of hemostasis. It should be noted that the thickness of fibrin fibers 3.1 times exceeded that in the group of intact animals (control). Also noteworthy are the results of an earlier study in which the thickness of fibrin fibers was inversely related to the intensity of thrombin generation [16, 17]. In particular, Collet J. P., et al. and Wolberg A. S. demonstrated *in vitro* that the enzymatic transformation of fibrinogen in the presence of low concentrations of thrombin occurs with the formation of 'thick' fibrin fibers with increased porosity of the resulting gel, whereas increased concentration of thrombin leads to the formation of 'thin' and short fibrin fibers characterized by relative resistance to fibrinolysis.

The traditional preparation for inactivation of warfarin (CPCF) demonstrated 6.7 times reduction of the volume of blood loss (in % of TBV) with reversal of INR (in prothrombin test) in comparison with use of placebo [11]. The above presented observations correlated with the morphologic picture of the wound surface in the form of *increased volume of thrombotic masses* (in comparison with the placebo group), the presence of thicker and frequently anastomosing fibrin fibers in their structure. In the thrombotic masses there was a high number of lysed erythrocytes which, as it is known, can on the one hand promote aggregation of platelets and form joint aggregates [18, 19], and, on the other hand, serve as donors of phosphatidyl serine providing a procoagulant surface for thrombin activation [20]. The presented combination of hemostasiologic, and, as a result, morphologic changes after use of CPCF promoted minimization of posttraumatic blood loss. Another histologic peculiarity was reduction of the number of platelets in large vessels near the wound surface (1.3 times in comparison with group 1 and 2.4 times — with group 2). Probably, platelets, being important participants of hemocoagulation, were more actively involved in thrombosis in the presence of CPCF.

The absence of the relationship between hemostasiologic changes in venous blood (systemic circulation) *with the underlying use of exogenous FM* (0.25 mg/kg), *and morphologic changes in the wound was demonstrated*. Thus, replacing of CPCF with FM was not accompanied by signs of compensation of warfarin-induced coagulopathy (according to INR, thromboelastometry and calibrated thrombography). Nevertheless, the use of the given fibrinogen derivative led to a sharp reduction of blood loss (9.1 times in % of TBV as compared with placebo) [11]. In histologic evaluation, the use of FM was associated with formation of comparatively more massive thrombotic deposits in the wound area. Here, we also observed the most active involvement of both lysed erythrocytes and platelets in the process of local thrombosis, and the latter were more actively involved in the observed process.

CONCLUSION

The conducted study showed that the externally introduced fibrin monomer can produce a local hemostatic effect in the conditions of dosed experimental trauma and coagulopathy induced by taking warfarin. Hemostatic effect was mediated by intense thrombosis on the wound surface of the liver with active involvement of platelets in the process. With this, no effect of fibrin monomer on systemic hemostatic reactions in venous blood was found. In pour opinion, the demonstrated effects of fibrin monomer may be mediated by mechanisms of action of this fibrinogen derivative not yet clarified, which necessitates continuation of research in this direction.

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