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Assessment of the effect of exogenous fibrin monomer on post-traumatic bleeding in hypofibrinogenemia caused by administration of snake venom *Agkistrodon rhodostoma*

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Abstract

Aim. To assess the effect of fibrin monomer on the rate of blood loss after controlled liver injury in hypofibrinogenemia induced by systemic administration of Malayan pit viper venom (*Agkistrodon rhodostoma*).

Methods. A placebo-controlled study of the hemostatic effect of fibrin monomer administered intravenously at 0.25 mg/kg, and coagulation parameters in the controlled liver injury with profound hypofibrinogenemia caused by administration of Malayan pit viper venom was conducted in 34 male Chinchilla rabbits. The distribution of the studied parameters was investigated by the Shapiro–Wilk test. Statistical differences between groups were tested by Student's t-test, Mann–Whitney U test, or Wilcoxon test, as appropriate. Differences in mortality rate were examined using Fisher's exact test.

Results. A model of experimental toxogenic disseminated intravascular coagulation was reproduced, manifested by high mortality of animals (50.0%), severe blood loss (increased blood loss by 1.78 times), hemolysis, a decreased platelet count (by 19.6% of median) and platelet dysfunction, fibrinogen consumption (protein content less than 0.9 g/l), hypocoagulation as well as intensive D-dimer production (increased concentration by 25.0 times of median). A high level of the fibrin derivative demonstrated activation of fibrin formation and fibrinolysis in the bloodstream of the animals. Systemic prophylactic administration of exogenous fibrin monomer after receiving snake venom did not lead to a decrease in post-traumatic bleeding, whereas earlier, during reproduction of disseminated intravascular coagulation caused by streptokinase infusion, such a hemostatic effect of fibrin monomer was shown.

Conclusion. The absence of fibrin monomer effect (at a dose of 0.25 mg/kg) on the severity of blood loss in toxogenic disseminated intravascular coagulation may be associated with more profound disseminated intravascular coagulation and a sharp 25-fold increase in D-dimer levels that can act as a fibrin monomer polymerization inhibitor.

Keywords: hemostatic system, snake venom, fibrin monomer, liver injury, hemostatic effect, rabbits.

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Background. Post-traumatic blood loss is one of the serious complications of invasive interventions and other conditions. The rate and volume of such blood loss is largely determined by the number and activity of platelets, as well as the fibrinogen concentration, which is significant in hemostatic reactions in relatively large caliber vessels. It is synthesized by the liver cells, and its blood plasma concentration significantly exceeds the level of other coagulation factors [1,2].

Fibrinogen represents a dimeric protein consisting of three pairs of polypeptide chains (Aα, Bβ, and γ). After the tissue damage and initiation of hemocoagulation, thrombin separates fibrinogen into two pairs of fibrinopeptides (A and B), and a fibrin monomer (FM) is formed which combines

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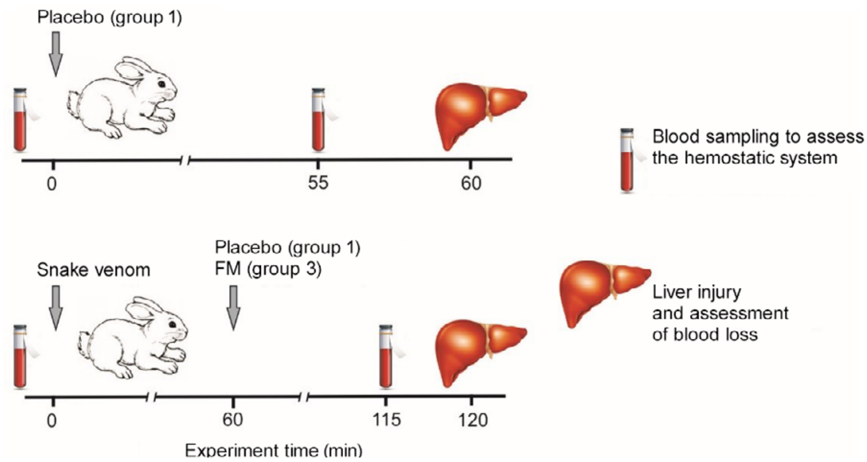

Figure 1. Design of experiments with dosed liver injury; fibrin monomer (FM).

with similar molecules to form insoluble fibrin polymers [3,4]. At the same time, thrombin activates factor XIII, resulting to blood clot stabilization and relative plasmin resistance [5].

Products of fibrin degradation is commonly known to be represented by a wide range of molecular weights when exposed to plasmin, including the lowest molecular weight degradation products of cross-linked fibrin in the form of DD/E complex [6]. The final product of plasmin digestion is the D-dimer fragment with approximately 195 kDa molecular weight, which is in complex with the E fragment (DD/E) in the circulation [7,8]. In addition, the D-dimer inherently regulates the final stage of blood coagulation by inhibiting the FM polymerization [2,3].

The fibrinogen level is most sensitive to blood loss and hemodilution, primarily resulting to depletion to a critically low level (<1.0 g/l) with extensive hemorrhages [9]. Hypofibrinogenemia is also possible with hyperfibrinolysis, usually concomitant with systemic thrombolytic therapy of arterial thrombosis and venous thromboembolic complications [10,11]. Hyperfibrinolysis and hypofibrinogenemia has been revealed to be associated with unfavorable outcomes in traumatic hemorrhages [12,13]. Experts recognized the significance of hypofibrinogenemia and its contribution to bleeding in patients who underwent cardiac surgery [14,15] and patients with acute leukemia [16], as well as in childbirth [17,18]. The fact is of interest, that the concentration of fibrinogen of 2.0 g/l and lower refers to independent risk factors for the development of major obstetric bleeding [19,20]. At the same time, the normal concentration of fibrinogen in the blood of women in late pregnancy increases to approximately 5.0 g/l.

In rare but indicative cases, fibrinogen depletion is registered in snake bites, which venom contains proteases capable of affecting this protein in the blood plasma, converting it into fibrin [21].

As previously demonstrated, experimental hypofibrinogenemia caused by thrombolytic therapy (with the use of streptokinase) led to severe post-traumatic hemorrhage; however, it was not noted with prophylactic systemic administration of FM [22]. With respect to the data obtained, it was of certain interest to assess the hemostatic effect of FM during defibrination associated with the administration of the Malayan pit viper venom (*Agkistrodon rhodostoma*) containing thrombin-like coagulase and a fraction with fibrinolytic activity [23]. A model of toxicogenic disseminated intravascular blood coagulation (DIC syndrome) was previously described by E. Regoeczi et al. [24].

This study aimed to examine the effect of FM on the intensity of blood loss after a dosed liver injury under conditions of hypofibrinogenemia induced by a systemic administration of the Malayan pit viper venom (*Agkistrodon rhodostoma*).

Materials and methods. An experimental study was conducted on 34 healthy sexually matured male chinchilla rabbits weighing 3.0–4.5 kg, the most appropriate type of biological object for this kind of study [25]. Prior to the experiment, subjects were kept under standard conditions in accordance with SP 2.2.1.3218–14 "Sanitary and epidemiological requirements for the arrangement, equipment, and maintenance of experimental biological clinics (vivaria)."

Subjects were divided into three groups using a random number generator. The study design is presented in Fig. 1.

An aqueous solution of 0.5 ml placebo (3.75 M urea solution corresponding to its concentration in FM solution) was injected to subjects of group 1 (n = 13) into the marginal ear vein (intravenously) using a Cathy catheter needle. An aqueous solution of the Malayan pit viper *Agkistrodon rhodostoma* venom (Siberian Serpentarium, Novosibirsk) was similarly injected to the subjects of groups 2 (n=10) and 3 (n = 11) at a dose of 5.0 μg/kg (snake venom is in crystalline state, prepared from raw materials after drying in a sealed desiccator in the presence of the dehydrated silica gel, with a moisture content of 91.8%).

One h after snake venom administration, 0.5 ml of placebo or an aqueous solution of FM at a dose of 0.25 mg/kg was injected intravenously to the subjects of groups 2 and 3.

One h after the placebo or FM solution administration to previously anesthetized animals (general anesthesia with telazol [Zoetis, Russia], intravenously at a dose of 10 mg/kg), a dosed liver injury occurred in accordance with current recommendations [26], which are described in greater detail in our previous study [27]. Afterwards, using a sterile gauze wipe, the nature of parenchymal bleeding was assessed by the calculated volume of blood loss as a percentage of the circulating blood volume (%CBV), taking into account the subject's body weight [28] and the rate of blood loss per time unit (mg/s) [25,26].

The experiment was terminated during the cessation of bleeding from the wound or after the cardiopulmonary arrest of the subject (death). The surviving subjects were sacrificed by an overdose of anesthetics [29].

To study the hemostatic system, blood was obtained from the marginal ear vein (by gravity) after phlebotomy, while rejecting the first drops. This procedure was performed twice, immediately before intravenous administration and 55 min after FM or placebo injection before causing dosed liver injury (Fig. 1). The blood obtained was placed in tubes containing ethylenediaminotetraacetic acid to count the number of platelets and tubes with 0.11 M (3.8%) sodium citrate solution (with blood and stabilizer ratio of 9:1) to study the hemostasis system in accordance to Russian national recommendations [30].

Experiments on subjects were performed in accordance with the European Convention and directives for the protection of vertebrate animals used in experiment 86/609/EEC, as well as the Declaration of Helsinki and Rules for working with experimental animals. This study was approved by the local ethics committee of the Altai State Medical University (Protocol No. 12 of 11/12/2015).

The study of the hemostatic system includes the assessment of the following:

– Number of platelets in venous blood on the hematological analyzer Drew-3 (Drew Scientific Inc., UK-USA);

– Platelet function (by its aggregation induced by the disodium salt of adenosine diphosphate (ADP) taken at a concentration of 10 μM) on a Chronolog 490-2D aggregometer (CHRONO-LOG Corporation, USA);

– Activated partial thromboplastin time (APTT), prothrombin time (PT), thrombin time (TT) of clotting, as well as fibrinogen concentration on the Thrombostat 2 coagulometer (Behnk Electronik, Germany);

– The antithrombin III activity by the amidol method on a Photometer 5010 v5+ spectrophotometer (Robert Riele GmbH Co & KG, Germany); and

– Level of soluble FM complexes (SFMC).

Last 5 indicators were established using reagents manufactured by the Technologiya-Standard (Russia) in accordance with Russian recommendations [30].

Results of the assessment of APTT, PT, and TT were presented as a ratio calculated by the formula:

$$Ratio = CT_{experiment}/CT_{control}$$

where $CT_{experiment}$ is the coagulation time in experimental plasma (s) and $CT_{control}$ is the coagulation time in controlled plasma (s).

The level of blood plasma D-dimer was determined using the NycoCard® D-Dimer test system on a NycoCard Reader II reflectometer analyzer (Axis-Shield PoC AS, Norway). An integral method for studying the hemostasis system was used as thromboelastometry of citrate-stabilized blood using a ROTEM® Gamma thromboelastometer (Pentapharm GmbH, Germany) with a Startem reagent in the Natem mode.

The distribution of characteristics in the samples was assessed using the Shapiro–Wilk test. Depending on the distribution of characteristics, Student's *t*-test, Mann-Whitney U-test, or Wilcoxon's W-test were used. Differences in the level of lethality of subjects in groups were established using Fisher's exact test. Differences were considered statistically significant at $p < 0.05$. The experimental data were processed using the MedCalc Version 17.9.7 statistical program (license BU556-P12YT-BBS55-YAH5M-UBE51). Data obtained were presented as a median (*Me*), 25th and 75th percentiles (*Q*): Me $[Q_{25} \div Q_{75}]$.

Results. In the course of the study, a high mortality rate of subjects in group 2 (4/10) was revealed, due to cardiopulmonary arrest with continued bleeding, as well as great vessel thrombosis (1 subject). A mortality rate with similar frequency was registered in group 3 (6/11) due to blood loss and massive thrombogenesis (1 subject). Taking into account the absence of mortality in group 1, statistical significance of the intergroup difference

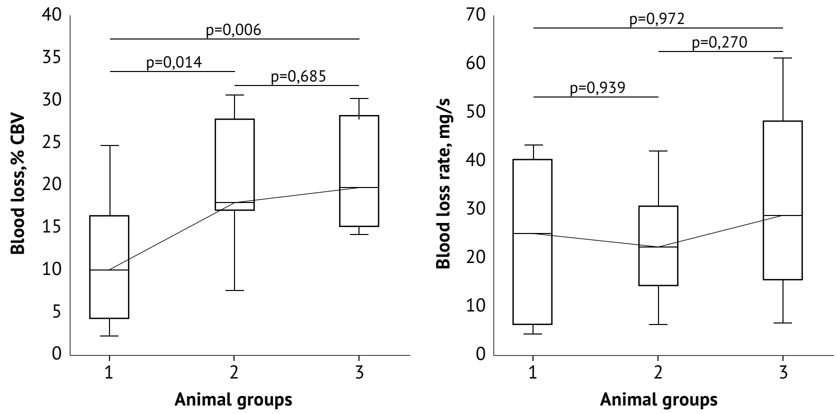

Figure 2. Comparative analysis of hemorrhagic manifestations of blood loss in experimental animals after dosed liver injury; circulating blood volume (CBV). Values are presented as median, represented by a horizontal line inside a rectangle, containing 50% of values obtained, and values corresponding to the 2.5 and 97.5 percentiles are represented by the lower and upper vertical bars.

for this indicator was p_{1-2}= 0.012 and p_{1-3}= 0.004. Note that the death of animals in groups 2 and 3 corresponded to the severity of blood loss (Fig. 2).

Thus, the volume of blood loss in the group of animals that received snake venom and placebo (group 2) was 1.78 times higher (18.0 [11.4÷25.3]% CBV), and in the group that received snake venom and FM (group 3), it was 1.96 times higher (19.8 [15.1÷27.7]% CBV) compared to the same indicator in the group of animals that received only the placebo (group 1; 10.1 [4.3÷16.3]% CBV). However, the rate of blood loss in subjects in these groups did not differ.

Along with the assessment of blood loss, a laboratory study of the hemostatic system was performed in the course of the study (Table 1). In accordance with the data obtained in subjects after administration of snake venom, the blood platelet count decreased (by 34.5% and 19.6% in groups 2 and 3 in comparison with the initial values in these groups by the median, respectively) and a noticeable decrease in their functional activity was also noted. In particular, the indicator of ADP-induced aggregation decreased (by the median) in group 2 by 3.1 times, and by 8.0 times in group 3.

Changes were combined with pronounced hypofibrinogenemia (<0.9 g/L in both study groups), an increase in the SFMC level (by 9.3 and 5.3 times, respectively), as well as a rapid increase in the D-dimer level (by 40.0 and 25.0 times, respectively), which indicated a marked proteolysis of fibrinogen and the generated fibrin. Disorders revealed were accompanied by a moderate but statistically significant decrease in the antithrombin III activity, one of the main physiological anticoagulants.

Accordingly, in the context of platelet dysfunction and hypofibrinogenemia, results of both screening tests (APTT, PT, and TT) and thromboelastometry of citrate-stabilized blood were expected. For all the indicators studied, a pronounced hypocoagulation shift was noted, and in some cases, plasma or blood incoagulability was observed (see Table 1).

Discussion. In the course of the study, characteristic manifestations of the experimental toxicogenic DIC syndrome were observed as described by Z.S. Barkagan et al. [31]. Manifestations include high mortality rate, tendency for severe blood loss, hemolysis, and decreased platelet count and dysfunction, as well as consumption of fibrinogen and to a lesser extent, antithrombin III. Changes were combined with intensive synthesis of D-dimer, demonstrating the activation of processes of fibrin formation and fibrinolysis in the bloodstream of the subjects.

It can be noted that the systemic administration of 0.25 mg/kg of exogenous FM after the subjects received snake venom did not lead to a decrease in post-traumatic blood loss. At the same time, we previously described such a hemostatic effect of FM during streptokinase-induced defibrination [22]. Results of this study reveal that the prophylactic use of FM (in comparison with placebo) reduced both mortality rate (from 36% to 6%) and the volume of blood loss associated with liver injury (%CBV from 15.4 [12.8 ÷ 19.2] to 1.4 [0.7 ÷ 4.4]; $p = 0.001$). A similar hemostatic effect was also found in the case of replacing FM with tranexamic acid (reference drug) administered intravenously at a dose of 15 mg/kg (blood loss as %CBV 1.0 [0.7÷2.3]; $p = 0.00004$).

The question, "what is the reason for the absence of hemostatic properties of FM when administered systemically to animals with toxicogenic DIC syndrome," is relevant. One of the reasons is believed to be a deeper hypofibrinogenemia registration in venom-associated defibrination (Table 1).

Table 1. Results of the assessment of the hemostasis system in groups of experimental animals.

Indicators	Group 1 (n = 13)		Group (n = 10)		Group (n = 11)	
	before placebo administration (1a)	after placebo administration (1b)	before AR and placebo administration (2a)	after AR and placebo administration (2b)	before AR and FM administration (3a)	after AR and FM administration (3b)
Platelet count, $\times 10^9$/l	477.5 [405.8 ÷ 621.5]	480.5 [412.3 ÷ 555.0]	611.0 [581.0 ÷ 640.0]	400.0 [378.0 ÷ 452.0]	542.0 [394.5 ÷ 635.0]	436.0 [412.0 ÷ 605.0]
		p_{1a-1b} = 0.151		p_{2a-2b} = 0.002 Δ 1.5 times		p_{3a-3b} = 0.483
ADP-induced aggregation, %	20.5 [19.0 ÷ 28.7]	22.0 [19.2 ÷ 31.4]	22.5 [20.9 ÷ 24.1]	7.4 [2.0 ÷ 13.0]	23.3 [17.7 ÷ 30.0]	2.9 [2.6 ÷ 8.3]
		p_{1a-1b} = 0.598		p_{2a-2b} = 0.008 Δ 3.1 times		p_{3a-3b} = 0.007 Δ 8.0 times
APTT, ratio	1.1 [1.0 ÷ 1.2]	1.1 [0.9 ÷ 1.2]	1.1 [1.1 ÷ 1.2]	1.1 [0.9 ÷ 1.1]	1.1 [1.0 ÷ 1.3]	1.2 [1.1 ÷ 1.6]
		p_{1a-1b} = 0.248		in 3 cases n.r. within 3 min		in 5 cases n.r. within 3 min
PT, ratio	0.9 [1.0 ÷ 1.2]	0.9 [0.9 ÷ 1.1]	1.1 [0.9 ÷ 1.1]	0.4 [0.3 ÷ 0.5]	1.0 [0.9 ÷ 1.2]	0.4 [0.3 ÷ 0.4]
		p_{1a-1b} = 0.476		in 3 cases n.r. within 2 min		in 5 cases n.r. within 2 min
TT, ratio	1.0 [0.9 ÷ 1.0]	0.9 [0.9 ÷ 1.0]	1.1 [1.0 ÷ 1.1]	1.2 [0.8 ÷ 1.4]	0.9 [0.9 ÷ 1.2]	—
		p_{1a-1b} = 0.215		in 6 cases n.r. within 2 min		in 9 cases n.r. within 2 min
Fibrinogen, g/l	3.3 [2.8 ÷ 4.4]	3.7 [2.8 ÷ 4.5]	3.3 [3.1 ÷ 3.9]	<0.9	3.1 [2.5 ÷ 3.7]	<0.9
		p_{1a-1b} = 0.811		p_{2a-2b} = 0.0001		p_{3a-3b} = 0.008
SFMC, mg/100 ml	3.0 [3.0 ÷ 4.5]	3.0 [3.0 ÷ 4.0]	3.0 [3.0 ÷ 8.5]	28.0 [26.0 ÷ 28.0]	4.5 [3.8 ÷ 5.8]	24.0 [21.5 ÷ 28.0]
		p_{1a-1b} = 0.866		p_{2a-2b} = 0.0001 9.3 times		p_{3a-3b} = 0.005 Δ 5.3 times
D-dimer, mg/ml	100.0 [100.0 ÷ 100.0]	100.0 [100.0 ÷ 200.0]	100.0 [100.0 ÷ 187.5]	4000.0 [2150.0 ÷ 5500.0]	200.0 [100.0 ÷ 200.0]	5000.0 [2500.0 ÷ 5650.0]
		p_{1a-1b} = 0.201		p_{2a-2b} = 0.003 Δ 40.0 times		p_{3a-3b} = 0.008 Δ 25.0 times
Antithrombin III, %	100.0 [94.0 ÷ 103.8]	104.0 [96.3 ÷ 104.8]	104.0 [99.8 ÷ 105.8]	95.0 [92.0 ÷ 100.0]	100.0 [95.0 ÷ 109.0]	92.0 [84.0 ÷ 95.5]
		p_{1a-1b} = 0.286		p_{2a-2b} = 0.002 Δ 1.1 times		p_{3a-3b} = 0.037 Δ 1.1 times
Thromboelastometry indicators						
CT, s	605.5 [453.8 ÷ 801.5]	628.0 [479.0 ÷ 856.0]	479.0 [333.3 ÷ 627.5]	1100.0 [349.0 ÷ 2220.0]	420.0 [345.0 ÷ 452.5]	900.0 [459.0 ÷ 2428.5]
		p_{1a-1b} = 0.821		p_{2a-2b} = 0.049 Δ 2.3 times		p_{3a-3b} = 0.022 Δ 2.1 times
Angle α, degrees	57.0 [46.5 ÷ 62.0]	55.0 [49.0 ÷ 65.0]	73.5 [66.3 ÷ 78.0]	63.0 [62.0 ÷ 64.0]	73.0 [71.0 ÷ 76.0]	33.0 [30.5 ÷ 49.5]
		p_{1a-1b} = 0.207		in 5 cases n.r.		in 8 cases n.r.
MCF, mm	59.5 [56.0 ÷ 64.3]	58.0 [54.0 ÷ 64.0]	60.0 [58.3 ÷ 61.8]	25.0 [22.0 ÷ 29.0]	67.0 [62.0 ÷ 68.5]	10.5 [6.3 ÷ 22.3]
		p_{1a-1b} = 0.956		in 4 cases n.r.		in 5 cases n.r.
A10, mm	44.0 [40.8 ÷ 52.5]	43.0 [39.0 ÷ 50.0]	51.0 [41.5 ÷ 59.5]	19.0 [7.8 ÷ 31.5]	61.0 [54.5 ÷ 62.0]	13.0 [6.0 ÷ 16.0]
		p_{1a-1b} = 0.422		in 5 cases n.r.		in 6 cases n.r.

Note: *p*, level of statistical significant differences between the compared Indicators; n, number of subjects in the group; AR, venom of snake *Agkistrodon rhodostoma*; ADP, adenosine diphosphate; FM, fibrin monomer; APTT, activated partial thromboplastin time; PT, prothrombin time; TT, thrombin time; SFMC, soluble fibrin-monomeric complexes; CT, starting time of coagulation; angle α, clot amplitude; MCF, maximum clot hardness; A10, clot amplitude after 10 min; Δ, difference in indicators; n.r., no registration (not registered).

The fibrinogen concentration decreased less noticeably 2 h after the administration of streptokinase (150,000 IU/kg), from 3.0 (2.1 ÷ 3.6) to 2.3 (1.8 ÷ 3.0) g/l, or by 23.3%; moreover, an increase in the level of D-dimer was not observed, which can be easily explained by streptokinase-induced fibrinogenolysis.

Therefore, it is necessary to pay special attention to the last marker, since the blood plasma level in the venom model increased 25 times, as shown in results, compared to the initial level (before administration) (Table 1). D-dimer is known to be capable of interacting with FM, blocking their polymerization with the formation of SFMC or the so-called "blocked fibrinogen" [2, 32]. This phenomenon was noted due to a significant increase in the SFMC level in the blood plasma of the subjects that received snake venom, namely in groups 2 and 3 (Table 1).

Finally, a decrease in platelet aggregation activity in animals of these groups can be noted, which is also apparently associated with D-dimer overproduction, which, at first glance, in itself can result in severe post-traumatic blood loss, regardless of exogenous FM administration. However, in our case, this is not entirely true, as indicated by results of earlier experiments with pharmacologically inhibited platelet function and prophylactic (before injury) administration of exogenous FM [33].

Conclusion. It can be assumed that the low hemostatic activity of FM *in vivo*, taken at a dose of 0.25 mg/kg, in a toxicogenic model of DIC syndrome is primarily due to the fibrinolysis and a concomitant increase in the D-dimer level. For the hypothesis confirmation, further experiments are planned to be conducted with sequential systemic administration of purified preparations of D-dimer and FM to animals.

Author contributions. A.P.M. was the work supervisor; V.M.V. and I.I.Sh. created the concept and design, formulated the experimental model, performed the data analysis and interpretation, validated essential intellectual content, and granted the final approval of the manuscript for publication; D.A.O., N.A.L., and D.A.M. set up an experimental model and were involved in data analysis and interpretation.

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Conflict of interest. Authors declare no conflict of interest.

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