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Hemostatic Activity Of The Mucus Of The Skin Of Fish.

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ABSTRACT

There is fragmentary information about the presence of high hemostatic activity of the native mucus of the skin of fish in relation to mammalian hemostasis in vitro. In order to verify this opinion, the haemostatic properties of the mucus of the skin of fish were evaluated. It was established that the clotting time of whole blood of sheep under the influence of mucus was 3.36±0.03 min versus 8.39±0.15 min in the control. When assessing the effect of mucus on plasma hemostasis of sheep, no reliable indicators were obtained. When using the mucus of the skin of fish as a sheep platelet agonist, an aggregation index of 102.9±10.28% was obtained against 19.2±2.36% in the control, the aggregation rate was 0.01±0.004 min versus 0.12±0.02 min control. At the same time, platelet aggregates formed under the action of mucus were equally stable in both the control and experimental groups. The platelet disaggregation index was 9.13±1.22% versus 9.09±0.41%, respectively. With the help of an immunoassay, the active hemostatic components of prothromboplastin and thromboplastin, blood coagulation initiators, were found in the composition of the mucus of the skin of fish. The results of the studies confirmed the high hemostatic activity of the mucus of the skin of fish and argue for the need for further in-depth study of its composition in order to create on its basis selective correctors for the hemostatic system in humans. A very promising development of biological hemostatic based on the mucus of the skin of fish. This will help solve the problem of bleeding in surgery. Economically, this drug will be very beneficial, because the mucus of the skin of fish is a cheap renewable resource, a rich source of protein components, and the demand for the drug in medicine will be very high.

Keywords: fish, mucus, blood, aggregation, platelets, coagulation, hemostasis.

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INTRODUCTION

Studies of various aspects of the hemostasis system in various biological objects [1, 2] and man [3, 4] are of great practical importance, since it opens up possibilities for regulating the state of the body in adverse conditions [5, 6]. In addition, the identification of patterns of work of the hemostasis system and the continued search for substances affecting it can increase the success of many therapeutic measures in humans [7] and animals [8,9]. The application of this knowledge in practical biology has already given a tangible effect, proving the need to continue research in this area [10].

In veterinary and medical surgical practice, drugs based on biological tissues are most often used to stop bleeding. To date, there are several projects whose purpose is to create a universal biological glue that can stop the bleeding and heal the wound. Clay MeTro (Australian University, Sydney) is tested and used in surgical practice, based on protoelastin protein molecules, which can glue up a wound even on the lung, and eventually speed up its healing [11]; DERMABOND - medical adhesive for local use (ClosureMedicalCorp (ETHICON, INC.)), Which includes a monomeric (2-octyl cyanoacrylate) substance [12]; CryoLife, Inc. BioGlue® biological adhesive (USA), based on plasma albumin bull [13].

Preparations based on biological tissues of fish and crustaceans have found wide application. Thus, due to the high content of tissue thrombokinase and kinins (coagulation factor) in the skin mucus, a high rate of blood coagulation is provided when fish are injured. Chitosan-based drugs are actively used to stop bleeding in medical and military practice [14]. Dressings using thrombin and salmon fibrinogen successfully stopped both aortic and superficial wound bleeding, and the results of this study showed that repeated exposure to plasma proteins of salmon for six months did not give any adverse effects from the immune system [15]. In the Russian North-Eastern Federal University named after M.K.Ammosov is conducting research on the creation of a medical biological glue based on a sturgeon swim bladder rich in collagen [16]. However, despite all the advantages of these drugs, their common drawback remains the high cost of maintaining and growing hydrobionts, as well as the difficulty of obtaining components for the manufacture of these drugs.

At present, there is no information on the availability of hemostatic preparations on the pharmaceutical market based on the active components of fish skin mucus. However, earlier on the basis of the analysis of blood coagulation in the White Sea fish - cod, navaga, catfish, gobies and pinagora, it was concluded that their "biochemical blood coagulation system is similar to the system of mammals." The main thrombogenic protein components have been found in fish: thrombotropin, prothrombokinase and thrombokinase, prothrombin, thrombin and fibrinogen. They also found that the slime of the skin of fish is a rich source of prothrombokinase. Mucus from the skin of cod, even diluted 10 times, is capable of coagulating oxalate plasma within 12–13 s [17, 18].

The results of previous own studies also demonstrate the potentially high hemostatic activity of the native mucus of the skin of fish in relation to the mammalian hemostasis system in vitro [19,20].

In this regard, the purpose of our study was to assess the hemostatic activity of the mucus of the skin of fish as a possible basis for a biomedical drug to activate blood coagulation.

MATERIALS AND METHODS

The research was conducted in strict accordance with ethical principles established by the European Convent on protection of the vertebrata used for experimental and other scientific purposes (adopted in Strasbourg in March 18, 1986, and confirmed in Strasbourg in June 15, 2006) and approved by the local ethic committee of Federal State Budgetary Educational Institution of Higher Education "Vologda State Dairy Farming Academy by N.V. Vereshchagin", the local ethic committee of Joint Stock Company «Russian Research Center for Molecular Diagnostics and Therapy» and the local ethic committee of All-Russian SII of Physiology, Biochemistry and Animals' feeding.

All research work was carried out in two stages:

- assessment of the possibility of using mucus of the skin of fish to activate platelet aggregation and blood coagulation in vitro;

- determination of the presence of proteins in the mucus of the skin of fish - thromboplastin (coagulation factor III, tissue factor, TF) and prothromboplastin (coagulation factor XI, F11).

For the study used mucus from the skin of a number of fish species (carp - Cyprinus carpio Linnaeus, sturgeon - Acipenser gueldenstaedtii, beluga - Huso huso) grown in industrial conditions in the fish farm LLC RTF Diana of the Kadui district of the Vologda region. Mucus was obtained by the method of Schultz et al. (Schultz et al., 2007), where the mucus was collected in polyester sponges, cut into pieces of 2x2x1 cm. Fish were caught from the aquarium using a small net, placed intact side up and allowed to dry for 5 seconds, then performed with a sponge, removing approximately 30% of mucus from one side of the body. A sponge containing mucus was placed in a syringe, which was squeezed out until a sample of mucus escaped from the sponge, which was placed in a 1.5 ml Eppendorf tube. 1 ml of distilled water was added to a syringe with a sponge, which was then squeezed out to dissolve and remove the remaining mucus.

Experimental work in vitro was performed on the blood of healthy Romanov sheep. Blood sampling was performed from the jugular vein using BD Vacutainer® vacuum blood sampling systems (Dickinson and Company, USA). As a stabilizer of venous blood, a 3.8% solution of sodium citrate was used in a ratio of 9: 1. All tests were performed on whole blood, plasma enriched and platelet-poor. Samples of platelet-rich plasma were obtained by centrifuging citrated blood at 100g for 10 minutes, and platelet-free plasma at 300g for 20 minutes.

The effect of mucus on whole sheep blood was determined in the following way: one slide of a sheep was applied to one glass slide with an automatic dispenser in a volume of 100 µl, 50 µl of blood and 50 µl of fish mucus on a second glass slide, every 30 sec. determined by carefully tilting the glass, the formation of a dense clot. The accuracy of the method was evaluated on the basis of the results of repeated determinations of the hemostatic activity of mucus with the same portion of donor blood (at least 9 determinations).

The study of the effect of fish skin mucus in vitro functional platelet activity was performed using a KFK-2 photoelectric colorimeter (Russia) according to Howard M.A. The intensity of aggregation was assessed by the dynamics of changes in the light transmission of platelet-rich plasma when adding to it an aggregation inducer (ADP) and mucus of the skin of fish. The aggregating platelet aggregation index (SIPA), aggregation rate (SA), platelet aggregation index (IAP) and platelet disaggregation index (IPD) of sheep with fish mucus (experimental group) and the aggregation inducer - ADP in a concentration of 0.1 mg/ml (control group).

The effect of fish skin mucus on plasma hemostasis was studied in platelet-poor plasma. The resulting plasma was investigated on a single-channel coagulometer - THROMBOSTAT manufactured by Behnk Elektronik (Germany). The effect of mucus on the coagulation of blood plasma was determined by the following method: to the platelet-poor plasma in a volume of 0.1 ml, previously incubated for 60 seconds, fish skin mucus was added in an equal volume. As a comparison, the clotting time of platelet-poor plasma with thrombin was used (produced by Tekhnologiya-Standart, Russia).

Thromboplastin proteins (coagulation factor III, tissue factor, TF) and prothromboplastin (coagulation factor XI, F11) were determined by the presence of enzyme immunoassay (ELISA), immunohistochemistry (IHC) and dot-blot for 20 mucus samples from different fish species.

The ELISA was carried out in accordance with the instructions for the “Instruction manual” test kit (ELISA Cloud-Clone Corp.).

Conducting immunohistochemical analysis (IHH) was carried out as follows. On mucous glasses for IHC (uniformly over the entire surface), test specimens of mucus were applied in a volume of 50 µl, after which they were dried and fixed for 2 minutes in methanol. Then the glass was divided into two areas, one of which was not made alleged specific antibodies (at) - (negative control). AP (polyclonal, rabbit) were applied in a volume of 50 µl (in a dilution of 80-120 times the initial concentration declared by the manufacturer (Cloud-Clone Corp.)). The incubation was carried out in a humid chamber for 24 hours at 4 ° C. After that, the glasses were washed with 1 × PBS (phosphate-saline buffer with pH = 7.4). For the manifestation of immune replication, AP was added to rabbit Ig (rabbit anti-IgG) conjugated with horseradish peroxidase - 2F12-HRP and 3F1-HRP in the dilutions - 1: 200 (initial concentrations 0.8 and 0.6 mg / ml, respectively). Rabbit anti-IgG was added to the mixture in a 1: 1 ratio by volume. The incubation was carried out in a humid chamber for 24

hours at 4 ° C. After that, washing of the glasses with 1 × PBS was carried out. After washing, immunopreplication was developed with a solution of 3-aminoethyl-9-carbazole (AEC) prepared on a stabilized substrate buffer (0.3 ml of a concentrated solution of AEC was added to a substandard buffer diluted with deionized water 1: 9).

The dot blot was performed as follows. On the nitrocellulose membrane (NC), pre-cut into strips, put the test samples of mucus. After that, the NC was dried and placed in a 2% solution of bovine albumin (BSA) for 1 h at room temperature. Then, NC was washed in a wash buffer (0.05% Tween-20 solution in 1 × PBS) for 10 minutes. Then NTS were incubated in 1.5 ml (for each strip) of specific AP (polyclonal, rabbit) (at a dilution 80-120 times the initial concentration declared by the manufacturer (Cloud-Clone Corp.)) for 24 hours at 4 ° C. Then, washing was performed in wash buffer for 10 minutes. Then NTS were incubated in 1.5 ml (for each strip) AP to rabbit Ig (anti-rabbit IgG), conjugated with horseradish peroxidase - 2F12-HRP and 3F1-HRP, in dilutions - 1: 200 (initial concentrations 0.8 and 0.6 mg / ml, respectively). Rabbit anti-IgG was added to the mixture in a 1: 1 ratio by volume for 24 hours at 4 ° C. Then, washing was performed in wash buffer for 10 minutes. After washing, immunopreplication was developed with a solution of 3-aminoethyl-9-carbazole prepared on a stabilized substrate buffer (0.3 ml of a concentrated solution of AEC was added to a substandard buffer diluted with deionized water 1: 9). In the case of a positive reaction, the formation of an immune replica occurred, which looked like a dot (dota) ranging from pale to bright pink.

The results obtained during the study were processed using the software package Statistica 6.1. and using the Microsoft Excel software package. The values of the results obtained in the work are presented as an average value and standard error of the mean (M ± m). The data were compared using the Mann-Whitney U-test for independent groups. The value of P was taken equal to 0.05.

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RESULTS OF THE RESEARCH AND DISCUSSION

Due to the fact that fish live in the aquatic environment, they have developed many protective mechanisms in the process of evolution. The internal, external, and general pathways of the blood coagulation system of bone fish were first demonstrated in the works of foreign authors [21,22,23,24]. Studies conducted on teleost fish indicate that the coagulation process is fundamentally similar to the same process in other vertebrates, in particular in mammals [25,26,27,28,29,30].

According to modern concepts, 2 mechanisms are involved in stopping bleeding: vascular-platelet (primary) hemostasis and plasma-coagulation (secondary) hemostasis. However, it was found that secondary hemostasis in fish is still less active - such indicators as thrombin and prothrombin time in fish are 5-10 times longer than mammals [31,32,33,34], but at the same time, blood coagulation rate fish is much higher than that of mammals. This can be explained by the fact that the physiological role of secondary hemostasis in fish can be assumed by the skin mucus secreted by them.

Based on the results of earlier studies [17,18] that the main thrombogenic protein components were found in fish, and also that the skin mucus of fish is a rich source of prothrombo kinase, we conducted studies on the effect of fish skin mucus plasma hemostasis and sheep platelets.

Analyzing the results of the study, it can be noted that under the influence of the mucus of the skin of fish, whole blood clots faster (Table 1).

Table 1. Comparison of the clotting time of sheep's native blood and under the influence of carp skin mucus (Cyprinus carpio)

Indicators	Native blood (n=9)	Native blood + carp skin mucus (n=9)
Clot formation time, min	8.39 ±0.15	3.36 ±0.03*

* - Differences with native blood are significant, (p<0,05)

Thus, we can conclude that the mucus of the skin of fish has hemostatic properties. To establish the mechanism of hemostasis (primary or secondary), we studied the effect of mucus on platelet-poor sheep plasma (secondary hemostasis) and platelet-rich sheep plasma (primary hemostasis).

When studying the effect of mucus on the plasma-coagulation unit of sheep hemostasis, the results presented in Table 2 were obtained. As a comparison, the clotting time of platelet-poor plasma with thrombin was used.

Table 2. Clotting time of platelet-poor plasma sheep blood under the influence of activators (seconds)

Indicators	platelet-poor plasma + thrombin, (n=8)	platelet-poor plasma + carp skin mucus, (n=8)
Clot formation time, min	21.0±0.20	12.0±8.50

Note: no significant differences found

Analyzing the data in the table, it should be noted that there is no reliable activation of the plasma coagulation factors of the sheep under the influence of mucus (in some cases the plasma did not clot). Thus, the results obtained convince of the need for further study of the effect of fish mucus on plasma hemostasis.

To assess the effect of fish mucus on platelet activity, the aggregation activity of sheep platelets under the influence of mucus (experimental group) was determined in comparison with the effect of the ADP aggregation inducer (control group). ADP is an activator of platelets. When it is added to a platelet-rich plasma, aggregates are formed, the transparency of the plasma increases and, consequently, the flow of light passing through the cell increases. The results of determination of induced platelet aggregation in sheep of the control and experimental groups are given in table. 3

Table 3. Comparison of aggregation activity of fish mucus and ADP

Indicators	Units	Control group (n=10)	Experienced group (n=10)
SIPA	%	21.98 ±2.71	36.17±1.52*
SA	min	0.12±0.02	0.01±0.004*
IAP	%	19.2±2.36	102.92±10.28*
IPD	%	9.13±1.22	9.09±0.41

* - The differences are significant, ($p<0.05$)

To determine the presence of active hemostatic components in fish mucus - thromboplastin (coagulation factor III, tissue factor, TF) and prothromboplastin (coagulation factor XI, F11), we used enzyme immunoassay (ELISA), immunohistochemistry (IHC) and dot-blot.

Using the ELISA method, the presence of prothrombin protein in fish mucus was determined. In accordance with the recommendations of the manufacturer of the test kit, a calibration curve was constructed and mucus samples were analyzed.

According to the results, the obtained optical densities of mucus samples did not succeed in detecting the required protein using the AP of this ELISA kit, which indicates the absence of specificity of these antibodies to thromboplastin contained in the mucus of the skin of fish or the absence of this protein in the mucus. It should be noted that the mucus samples were subjected to ELISA analysis in the original (native) form, i.e. not diluted or affected by any other factors that could affect the physicochemical or biological properties of the test specimens.

Conducting IHC did not give pronounced results: the recording of the obtained data was difficult, and there was no clear formation of immune replication. In this regard, a dot-blot immunoassay was performed to confirm the results.

The obtained data on the results of the dot-blot indicate that all the samples of mucus (even when diluted several times) formed an immune replica with rabbit polyclonal antibodies specific for thromboplastin and prothromboplastin (there were pale or bright pink dots). all squares in which mucus samples were applied). The results obtained confirm the presence of the desired proteins in the composition of fish skin mucus.

CONCLUSION

Plasma-coagulative hemostasis of fish from mammals can be attributed to the fact that the skin mucus containing a very large amount of tissue thromboplastine coagulation, which plays the role of hemostatics, can assume the physiological role of secondary hemostasis in fish [18, 32]. Analyzing the results of the study of the hemostatic activity of the mucus of the skin of fish, we found that under its influence whole blood clots up faster. Thus, we can conclude that the mucus of the skin of fish has hemostatic properties. Apparently, mucus acts on plasma hemostasis, however, the results of the study have not yet found a reliable activation of plasma clotting factors of sheep under the action of mucus from the skin of fish. When using the mucus of the skin of fish as a platelet agonist, they obtained a significantly higher index and rate of aggregation than when using ADP, an active agonist of mammalian platelets. The resulting aggregates have the same stability as those formed under the action of ADP, which indicates the optimal structure of the aggregate and similar developmental mechanisms. In this regard, very practical work on the creation of a biological hemostatic on the basis of mucus skin of fish. Fish skin mucus is a cheap renewable resource and a rich source of protein components with significant hemostatic activity for both fish and mammals.

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