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Characterization of the biological effect of fish fibrin glue in experiments on rats: Immunological and coagulation studies

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Abstract

Fibrin glues (FG) of human or bovine origin are widely used for haemostasis and wound healing. In addition FGs are studied in many biomedical areas like cell therapy or tissue engineering. As any mammalian plasma products FG-s pose risk of transmission of bacteria, viruses, or prions and may compromise patient homeostasis. In this study, we examined coagulation parameters and immunological status of rats treated with salmon-derived FG. We evaluated the changes in thrombin time, prothrombin activity, and presence of antibodies on 46 Wistar rats. This study shows that salmon-derived FG, injected intraperitoneally, does not cause coagulation disturbances in the peripheral blood. After a first challenge with salmon-derived FG there were low but detectable amounts of antibodies revealed by ELISA and immunoblot. After a second administration there was substantial elevation of antibodies to FG components and other copurifying plasma proteins. Antibody reactivity to human Factor Va, revealed in three animals, was not associated with FG application. Taken together, blood immunological and coagulation parameters support the suitability of salmon-derived FG in the development of fibrin sealants for medical use.

Keywords

fibrin glue; coagulation; thrombin time; antibodies; animal experiments

INTRODUCTION

Fibrin glues (FG) of thrombin and fibrinogen composition were originally used to minimize blood loss during operations in clinical practice.¹ Current use and studies have expanded to cover many areas in biomedicine and reveal the potential of FG in cell therapy and tissue engineering.^{2–6} Studies have been conducted to assess FG as a potential controlled or extended release system for drugs. Fibrin, as a relatively slowly degrading biological scaffold can be loaded with active ingredients to achieve high local concentration of drugs

for controlled release.^{7–12} However, the main use and experimental research is still focused on haemostasis and wound healing in a variety of surgical settings. FGs currently approved for clinical use are derived from human or bovine plasma. As any plasma-derived product these materials pose potential risk for transmission of bacteria, viruses, or prions. This consideration limits widespread use of mammalian plasma-derived FG. Use of bovine proteins in FG may also compromise the patient homeostasis. Development of antibodies and their cross reactions to host coagulation proteins can severely alter normal haemostasis. For example, two thirds of reports about the occurrence of antibodies against coagulation factor V are due to previous bovine thrombin exposure.¹³

Our previous studies showed that Atlantic salmon blood proteins represent a potentially safer, equally effective, and less costly alternative to human or other mammalian blood proteins.

Coagulation and immunologic studies in a pilot study on rats and rabbits, treated intraperitoneally with salmon FG, showed no deleterious effects on coagulation profiles and no cross-reactivity with host fibrinogen or thrombin.¹⁴ Similarly, Ju et al. assessed the growth of mammalian neurons in bovine, human, and salmon fibrin and found that salmon fibrin gels encouraged neurite (dendrite and axon) growth to the greatest degree. Importantly, salmon FG was the most resistant to degradation by cellular proteases. It was suggested that salmon FG may be a beneficial scaffold for neuronal regrowth after central nervous system injury.¹⁵

In this study, we extend our previous experimental investigations aiming to assess the peripheral blood coagulation profiles and antibodies against FG components in enzyme-linked immunosorbent and immunoblot assays on rats intraperitoneally treated with salmon FG. We also studied possible cross-reactivity to human factor Va and determined C reactive protein levels to assess acute phase response before and after the intraperitoneal administration of salmon FG.

MATERIALS AND METHODS

Coagulation proteins

Salmon fibrinogen (Lot #1289) and thrombin (Lot #5031), purified as previously described were used for administration to experimental animals and as antigen in immunologic studies. Salmon fibrinogen was treated with 10 kGy gamma irradiation. Human Factor Va (Lot L6050564 C6050564) was obtained from US Biologicals (Massachusetts, MA).

Secondary antibodies, enzyme substrates, and other materials

Rabbit alkaline phosphatase-conjugated antirat IgG (A6066, Sigma, St. Louis) was used in combination with the enzyme substrates p-nitrophenyl phosphate (PNPP, Sigma, St. Louis, USA) at a concentration of 1 mg/ml in diethanolamine buffer, pH-9.8, in ELISA and 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) (B-8503 Lot 32K1430; N-6876 Lot 32K5307, Sigma-Aldrich, St. Louis, MO) in immunoblot assays. Microtiter Assembly Strip 1×8 enhanced binding, EB ELISA strip-plates (Cat no. 95029180, ThermoElectron OY, Finland) were used in ELISA and Trans-Blot® Transfer Medium Pure Nitrocellulose Membrane (NCM) at 0.2 µm (Control 13108, Bio-Rad Laboratories, CA) in immunoblotting throughout the studies. Swine serum was separated from clotted swine blood obtained from a local slaughterhouse by centrifugation.

Experimental animals

Forty-six Wistar rats (24 males, 22 females) were bred and kept at the local animal care facility (Biomedicum's Animal House, University of Tartu, Estonia). Rats were 11 months old, weighting 280–418 g (females) and 450–748 g (males) at the start of the experiment. Females and males were separately randomized in two groups, an experimental group that was treated with salmon fibrin and a control group treated identically with 0.9% sodium chloride in place of fibrin.

Experiments were approved by the local Ethical Committee for Animal Experimentation.

Blood sampling

Blood was taken from the tail vein in anaesthetized (50–70 mg/kg ketamine) rats by a 5-ml syringe containing 3.8% sodium citrate at a ratio of 1:9 (citrate: blood), immediately mixed by rotation, and centrifuged for 10 min at 2500g as described elsewhere.¹⁴ A portion of plasma was used for coagulation tests within 2–4 h and another portion was stored at –20°C for further antibody studies. Blood was taken at the start of experiment for baseline values and on days 11, 20, 30, 33, 42, and 46 (Fig. 1).

Administration of salmon thrombin/fibrinogen complex preparation

Salmon FG was prepared according to previous protocols.¹⁴ Totally, 11 mg of fibrinogen with 90 U of thrombin per kg body weight was injected intraperitoneally on the 2nd and 32nd day to the experimental groups of rats. The same volume of 0.9% sodium chloride was administered to animals in control groups (Fig. 1). The general status of animals was continuously monitored during the study. Special attention was paid to behavior of animals during and up to 6 h after injections.

Coagulation assays

For thrombin time (TT), prothrombin time (PT) and International Normalized Ratio (INR) evaluations in rats, human test kits developed by Diagnostica Stago (Asnieres, France) are readily usable when one follows rat blood specimen collection and treatment as described above.

TT in seconds was measured by the STA-thrombin kit (REF 00611 and REF 00669) and PT and INR by the STA-SPA 50 kit (for the determination of the combined factors II-VII-IX, i.e. prothrombin complex; REF 00105) using the STA Compact apparatus (Diagnostica Stago, Asnieres, France). TT was expressed in seconds and INR as the ratio of the measured clotting time to the mean normal clotting time raised to the International Sensitivity Index power of thromboplastin.

ELISA with salmon coagulation proteins

For detection of antibodies against salmon thrombin and fibrinogen we performed ELISA as described elsewhere.¹⁴ Briefly, after coating with salmon thrombin diluted with PBS (pH—7.4) overnight at +5°C (protein concentration 10 µg/mL) and blocking with 5% swine serum for 1 h at +37°C, the strips were incubated with rat plasma dilutions (1:100) in 1% swine serum in PBS for 1.5 h at +37°C. The strips were then incubated with rabbit alkaline phosphatase-conjugated antirat IgG (dilution 1:10,000) in 1% swine serum PBS for 1.5 h at +37°C followed by incubation with PNPP solution in diethanolamine buffer for 30 min. Optical densities (OD) at 405 nm of the wells as measures of relative amounts of reacting antibodies were evaluated using blank wells as baseline by spectrophotometry (Multiscan MCC/340, Labsystems OY, Finland).

For detection of antibodies against salmon fibrinogen we used the same procedure except with a different antigen concentration of 5 µg/mL and a plasma dilution of 1:5000.

ELISA for rat C-reactive protein evaluation

The Helica (tm) C-Reactive Protein Rat ELISA kit (Lot #121306, Helica Biosystems, Inc., Fullerton, CA) was used for measurement of rat plasma C-reactive protein (CRP) levels according to the manufacturer's instructions. Levels up to 600 µg/mL were taken for the normal range.

Reduced SDS-gel electrophoresis, Western blot

Reduced samples of salmon thrombin, fibrinogen, and human factor Va were applied on discontinuous SDS-polyacrylamide gels (5% stacking gel and 10% resolving gel). The gels were run in a MiniProtean II Electrophoresis Cell apparatus from Bio-Rad. The running buffer was 25 mM Tris-HCl (pH 8.3), 0.1% SDS, 192 mM glycine. The proteins were transferred onto NCM by semi-dry electroblotting in a 25 mM Tris-HCl buffer (pH 8.3), 192 mM glycine, 15% methanol at 50 V for 1 h using an Hoefer SemiPhor(tm) electro blot apparatus. After the transfer, molecular weight markers at 250, 150, 100, 75, 50, 37, 25, 20, 15, and 10 kDa (Precision Plus Protein (tm) standards of Bio-Rad; cat. 161-0363; L1610363) were stained with Amido Black dye and the rest of the NCM was blocked in a TBS-TM solution (TBS as 25 mM Tris-HCl buffer, pH 7.4, 150 mM NaCl; TBS-T as TBS + 0.05% Tween 20; TBS-TM as TBS-T + 3% nonfat dried milk solution) overnight at 5°C. The membrane was then incubated for 2 h with rat plasma diluted 1:500 in TBS-TM. Excess primary antibodies were removed by four successive washing steps using TBS-T. The rabbit alkaline phosphatase-conjugated antirat IgG antibodies were diluted 1:5000 in TBS-TM. After 1.5 h of incubation, four washing steps as above were performed followed by visualization with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT). The staining was stopped by washing the membrane several times in cold water.

Statistical analysis

Unpaired t-test was used for statistical analysis with R language and environment for statistical computing.¹⁶ p -values < 0.05 were taken as significant.

RESULTS

Effect of salmon FG administration on rat blood coagulation

Administration of two doses of salmon fibrin intraperitoneally had no effects on blood clotting in the experimental rat model. The results of coagulation tests are shown in Figure 2.

When changes in TT and INR in relation to each rat baseline values were analyzed, no differences in mean INR values between control group and experimental group rats were seen during the entire course of the experiment ($p > 0.05$). There are no differences in mean TT values before the first immunization and 10–30 days after the first treatment with salmon FG. After the second administration of FG, mean TT values in the experimental group were slightly lower and were statistically different from control group rats ($p < 0.001$), consistent with a nonspecific response to administration of a second dose of antigen. Clotting times normalized shortly thereafter, and coagulation tests on days 42 and 46 showed again no differences in mean TT times between control and experimental group rats ($p > 0.05$).

C-reactive protein levels in rat plasma

We evaluated C reactive protein levels in 10 randomly selected experimental and 10 control rats at baseline and on day 33 (24 h after the second FG administration) as CRP may reflect the development of an acute phase response shortly after FG administration to the rats. There were no abnormal CRP levels in any of the rat plasma samples tested.

Similarly, when changes in 33rd day plasma CRP values ($\mu\text{g/mL}$) relative to individual animal baseline levels were analyzed, no statistical differences were revealed between groups (Fig. 3).

Development of antibodies against salmon fibrinogen and thrombin

ELISA results showed that with either thrombin or fibrinogen as antigen all experimental rats after FG administration had significantly higher reactivity to these antigens than control rats. The highest values of optical densities reflecting antibody concentration were seen after the second immunization at the end of the experiment (Table I).

Antisalmon thrombin antibodies as detected by immunoblotting

When reduced salmon thrombin was stained with Amido Black one strong protein band representing salmon thrombin heavy chain (about 32 kDa) was evident (Fig. 4, lane 1). There was also another weak band seen at about 48 kDa and some other bands that represent plasma proteins copurifying in the process of salmon thrombin preparation, or more likely intermediate fragments formed during the activation of prothrombin.¹⁷ After incubating strips with experimental and control group plasma samples taken on the 46th day the main reaction was against the salmon thrombin heavy chain at 32 kDa. As expected, the highest ELISA OD values were seen with plasma samples showing strong reaction in the immunoblot (plasma from rats no. 2 and 10), and the lowest ODs with weak (plasma from rat. no. 7) or no (plasma from rat no. 19) reaction in immunoblot. Additional bands are seen with most plasma samples from the experimental group—at 70 kDa and/or 82 kDa. The intensity of these reactions was low, and 5 of 21 rats in the control group showed some reactions to a polypeptide at 82 kDa (Fig. 4).

Antisalmon fibrinogen antibodies as detected by immunoblotting

Fibrinogen is a dimer consisting of two $\text{A}\alpha$ chains, two $\text{B}\beta$ chains, and two γ -chains. The salmon fibrinogen $\text{B}\beta$ chain and γ -chain were easily verifiable by Amido Black staining whereas the $\text{A}\alpha$ chain was either not seen, because of heterogeneity or comigrated with the lower molecular weight band, as has been reported earlier (Fig. 5, lane 1).¹⁸ In addition to fibrinogen, a band at 220 kDa that stained with much less intensity is probably copurifying salmon plasma fibronectin which constitutes 3% of fibrinogen preparations unless it is specifically removed by affinity chromatography. Because fibronectin functions to improve cell adhesion to the clot, its retention in FG may be important to support the efficacy of FG in wound healing. After incubation of NCM strips with the 46th day plasma samples obtained from the experimental group (strips 2–4) and control group (strips 25–47), reactions against the salmon fibrinogen preparation were detected exclusively in the experimental group. The main reactions were against salmon fibrinogen $\text{B}\beta$ and γ -chains. In general, the fibrinogen antibody measurement results correlated well between ELISA and immunoblot assay (ODs over 1.0 in rats no. 2, 3, and OD below 0.2 in a rat no. 23).

Antihuman Factor Va antibodies as detected by immunoblotting

Staining with Amido Black for protein revealed reduced human Factor Va subunits at 75, 79, and 110 kDa (Fig. 6, lane FVa). After incubating strips with experimental group and control group 46th day rat plasma samples (altogether 46 plasma samples) we found

potential reactions to human factor Va in both experimental and in control groups. One of 23 rats from the experimental group showed antibody reactions against polypeptides at 75 and 79 kDa and one rat showed weak reaction against a 110-kDa polypeptide. At the same time one out of 23 control group rats also displayed reactivity to polypeptides at 75 and 79 kDa.

In these rats baseline and 11th-day plasma samples were studied side-by-side with 46th-day plasma samples. As seen from Figure 6 both control (rat no. 36) and experimental (rat no. 13) group rat plasma samples had antibody reactivity against polypeptides at 75 and 79 kDa throughout the whole study. In difference, rat no. 3 from the experimental group had a single reaction to 110 kDa polypeptide only on the 46th day of the experiment.

DISCUSSION

Several studies report risk of antibody development in patients exposed to topical bovine preparations.^{19–21} Patients receiving multiple exposures are eight times more likely to develop antibodies.¹⁹ Antibodies developed to fibrin glues derived from foreign proteins have the potential to cross-react with host coagulation proteins that could lead to haemostatic disturbances.

Our previous study of 14 rats and six rabbits examined stability, viral inactivation, coagulation efficacy, and immunologic parameters to demonstrate that salmon-derived fibrinogen and thrombin could be readily used as an alternative to bovine- or human-derived fibrin sealants for clinical applications. In addition, this study demonstrated that a salmon-derived fibrinogen and thrombin complex preparation does not cause impairments in peripheral blood coagulation among experimental animals.¹⁴

This study confirms the results of our previous investigations, but gives also important additional data about immunological inactivity of irradiated salmon FG preparation, confirming the safety of purification procedure itself. Since these results were obtained on a much larger group of experimental animals compared with what has been used in previous studies, we obtained a more solid basis to confirm our previous results supporting the safety of FG as tissue sealant.

In this study, we evaluated the changes in thrombin time (TT), prothrombin activity (INR), and presence of antibodies on 46 Wistar rats at the start of the experiment and on days 11, 20, 30, 33, 42, and 46 after two intraperitoneal challenges with salmon FG on days 2 and 32. Coagulation studies reveal no statistical difference in mean TT values between experimental and control groups before administration and up to 30 days after first administration of FG preparation. After the second administration of FG preparation a small decrease in TT values was transiently observed on day 33, and on days 42 and 46 the TT values between control and experimental group rats were indistinguishable again. These results are in good accordance with our previous findings. Mean INR values between control and experimental group rats in the present larger study show no differences during the entire experiment in variance with our earlier findings that suggest also a transient change at day 33.¹⁴ This difference might be related to the fact that in the earlier study data from only 7 experimental and 7 control animals were compared.

Immunoglobulin G type antibodies to both salmon-derived FG components were measured in rat plasma at the time points used for coagulation studies. ELISA and immunoblot tests showed that all rats in the experimental group that were challenged with salmon-derived FG complex preparation developed low amounts of antibodies to the salmon FG components after the first intraperitoneal FG application. After the second administration of FG, there was a substantial increase of antibody levels, either against salmon thrombin or fibrinogen. As development of the acute phase response could be expected after immunization of rats

(especially after the second administration of salmon FG) we also measured plasma CRP levels along with coagulation studies. However, we revealed no statistical differences between control group and experimental group rats' plasma CRP levels on day 33 relative to baseline levels, showing that no significant acute phase response occurred in connection with salmon FG application in rats.

Reduced salmon thrombin stained for protein after electrophoresis and transfer to NCM revealed salmon thrombin heavy chain (~32 kDa) as the main protein band. There were also some other bands with much less intensity representing lower protein content which are probably copurifying plasma products or thrombin fragments. After immunoblot analysis with experimental and control group 46th day rat plasma, reactions against salmon thrombin heavy chain were detected in the experimental group. The intensity of antibody reactions to polypeptides other than thrombin is low, and in particular reactivity to Factor V was not seen in any sample. Reactions to salmon thrombin heavy chain were not seen in the control group, but 5 of 23 rats tested showed low reactivity to a polypeptide at 82 kDa. Higher OD values in ELISA correlated well with higher number and stronger reactivity patterns in immunoblot assays with either antigen, showing that ELISA appropriately represents animals' immune reactivity to FG. Accordingly, ELISA results could be used as a measure of the host's general immune reactivity against salmon FG. Nevertheless, ELISA does not give information about the development of antibodies against specific components of FG, most importantly about potential contamination of FG purification by-products that may be highly immunogenic for the host.

Electrophoreses and transfer to NCM of reduced salmon fibrinogen revealed by Amido Black staining on NCM salmon fibrinogen Bb chain and γ -chain as the main constituent of the preparation. The A α chain could not be separated possibly because of its anomalous electrophoretic mobility or a slight degradation.¹⁸ A few other protein bands were seen with much less intensity as seen also in thrombin preparation which are likely to be copurifying plasma products. In immunoblotting of experimental rats treated with salmon fibrinogen preparation, 46th day plasma samples revealed reactive bands against salmon fibrinogen Bb chain (~54 kDa) and γ -chain (~49 kDa), and against a 220 kDa protein that is probably copurifying salmon plasma fibronectin.

However, in the last decade most clinical cases where Factor V antibodies were reported were associated with bovine thrombin exposure.¹³ We aimed to study the appearance of autoantibodies to human Factor V as a result of salmon FG administration. To test possible Factor V contamination in salmon FG and its possible immunogenicity we used human Factor Va as an antigen in immunoblots using rat plasma samples obtained at day 46. A very low level of positive reactions to antigens in the Factor V preparation was revealed in both experimental and control groups. Most importantly, these reactions were also seen with plasma samples taken before the experiments and on day 11, except for one experimental rat showing reactivity to 110 kDa protein only on day 46. Importantly, in no case was there a change in coagulation times even in animals that showed potential reactivity.

Another important finding, although done in a smaller number of control and experimental animals, is the absence of CRP increase after a second administration of salmon FG. This result shows that in addition to its relatively inert nature revealed in blood coagulation studies, salmon FG is also a weak stimulator of innate immunity and acute phase protein liberation. These studies will be continued in a larger group of animals evaluating additional humoral factors of innate immunity (complement system, etc.) in parallel.

CONCLUSIONS

In concordance with our previous results this extended study on 46 rats shows that salmon-derived FG, injected intraperitoneally, does not cause coagulation disturbances in the peripheral blood. Only a slight, transient drop in TT was revealed after a second intraperitoneal administration of salmon FG that resolved within the next few days. After a first challenge with salmon fibrinogen and thrombin there were low but detectable amounts of antibodies revealed by ELISA and immunoblot. After a second administration there was substantial elevation of antibodies to fibrin components and other copurifying plasma proteins. However, antibody reactivity to human Factor Va was not associated with FG application. Enzyme-linked immunosorbent assay and immunoblot results were in good correlation and therefore both assays are appropriate to use for the detection of antibodies in response to FG administration. However, the immunoblot assay is suggested for use in order to determine the development of antibodies against possible contaminants of FG.

Taken together, blood immunological and coagulation parameters support the continued study of salmon FG in the development of fibrin sealants for medical use.

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Figure 1.
Schematic view of immunization and bleeding protocol.

Figure 2. Thrombin time and INR as measured in plasma samples taken before and after intraperitoneal administration of salmon fibrin on days 2 and 32. Solid symbols represent mean values of data control animals not treated with fibrin and open symbols are mean values from animals treated with salmon fibrin. Error bars denote standard deviation from mean value based on data from 23 rats in either study group.

Figure 3. Changes between baseline and 33rd day plasma CRP values in control and experimental group rats.

Figure 4. Immunoblot with salmon thrombin in experimental group (strips 2–24) and control group (strips 25–45) rats using plasma samples taken on the 46th day. M, molecular weight markers; 1, salmon thrombin lot#5031, stained with Amido Black.

Figure 5. Immunoblot with salmon fibrinogen in experimental group (strips 2–24) and control group (strips 25–47) rats using plasma samples taken on 46th day. M, molecular weight markers; 1, salmon fibrinogen lot#1289, stained with Amido Black.

Figure 6.

An example of the antibody reactivity towards human factor Va preparation as revealed by immunoblot using plasma samples from rats at baseline, 11th and 46th day of the experiment. Rat no. 36 belongs to the control group and rat no. 13 and 3 to experimental group. MWM, molecular weight markers; FVa, human factor Va, stained with Amido Black.

TABLE I

Average Antithrombin and Antifibrinogen Antibody Levels (Showned as OD Values) in Control and Experimental Rats Groups as Evaluated by ELISA from Blood Samples Taken During Whole Experiment Period

Bleeding Time	Control Group of Rats	Experimental Group of Rats	<i>p</i> -Value
Antithrombin antibody results in OD units (mean $\pm$ SD)			
Baseline	0.075 ($\pm$ 0.007)	0.077 ($\pm$ 0.010)	$p = 0.17$
Day 11	0.071 ($\pm$ 0.007)	0.088 ($\pm$ 0.011)	$p < 0.01$
Day 20	0.073 ($\pm$ 0.010)	0.136 ($\pm$ 0.044)	$p < 0.01$
Day 30	0.078 ($\pm$ 0.014)	0.189 ($\pm$ 0.071)	$p < 0.01$
Day 33	0.080 ($\pm$ 0.012)	0.167 ($\pm$ 0.069)	$p < 0.01$
Day 42	0.096 ($\pm$ 0.047)	0.441 ($\pm$ 0.184)	$p < 0.01$
Day 46	0.077 ($\pm$ 0.014)	0.472 ($\pm$ 0.203)	$p < 0.01$
Antifibrinogen antibody test results in OD units (mean $\pm$ SD)			
Baseline	0.063 ($\pm$ 0.003)	0.063 ($\pm$ 0.003)	$p = 0.48$
Day 11	0.063 ($\pm$ 0.004)	0.167 ($\pm$ 0.065)	$p < 0.01$
Day 20	0.068 ($\pm$ 0.019)	0.314 ($\pm$ 0.168)	$p < 0.01$
Day 30	0.076 ($\pm$ 0.025)	0.353 ($\pm$ 0.183)	$p < 0.01$
Day 33	0.070 ($\pm$ 0.010)	0.226 ($\pm$ 0.148)	$p < 0.01$
Day 42	0.104 ($\pm$ 0.068)	0.850 ($\pm$ 0.247)	$p < 0.01$
Day 46	0.062 ($\pm$ 0.002)	0.788 ($\pm$ 0.231)	$p < 0.01$