Effect of Glass Contact on the Clotting Time of Carp Blood

Hiroshi Kawatsu* and Makoto Sato*

(Accepted January 6, 1987)

Clotting times of carp blood were determined using three kinds of test tubes: glass, silicone treated glass and polystyrene tubes. In order to eliminate the effect of glass contact, test plasma was prepared using tools made of plastic. When determining the recalcification time (RT), clot formation was observed only in the glass tubes and no clot was formed in either the silicone treated glass or the polystyrene tubes. Plasma in contact with glass beads clotted in polystyrene tubes and the clotting time was shortened when the amount of added glass beads was increased. Significant differences were also observed in partial thromboplastin time (PTT) and prothrombin time (PT), while no noticeable differences were observed in activated partial thromboplastin time (APTT). These findings suggest that carp blood has a XII-like factor which is converted to the activated form by contact with glass or ellagic acid.

In a previous paper,¹⁾ we reported that activated partial thromboplastin time (APTT) is very short and the intrinsic coagulation system is developed in carp blood. This finding led us to suppose that the activity of a contact factor, such as factor XII in mammalian blood is high in carp blood, and if such is the case, it is essential to control the effect of this factor when measuring the extrinsic system. Glass, celite, kaolin and ellagic acid are known to be involved in the activation of factor XII in mammalian blood. As we used glassware in the measurements of clotting time in the previous study, it was necessary to evaluate the effect of glass contact on the blood coagulation.

The present paper describes the effect of glass contact on the clotting time of carp blood and gives evidence of the existence of the contact factor.

Materials and Methods

Test Animals

Common carp *Cyprinus carpio* was used for the experiments. Fish were obtained from a private fish farm and kept in the ponds until used.

Preparation of Citrated Plasma, Serum and Adsorbed Plasma

Citrated plasma: In order to avoid possible activation of the contact factor due to glass contact, plasma was prepared using tools made of plastic. Needles of syringes were coated with

KF 96 silicone (Shin-etsu Chemical Co., Ltd., Tokyo). Nine parts of the blood were withdrawn into a syringe containing one part of 3.13% trisodium citrate solution. Plasma was collected by centrifuging blood at 3,000 rpm for 5 min and kept at 4°C until tested.

Stored serum: Clotted blood was left at room temperature for 1 h and was centrifuged at 3,000 rpm for 5 min. Three or four serum samples were pooled and stored at 4°C for 2 or 3 days until used.

Adsorbed plasma with BaSO₄: Barium sulphate was added to the pooled citrated plasma (200 mg/ml plasma) and stirred with a plastic rod for 30 min at 25°C. After centrifugation at 3,000 rpm for 5 min, the supernatant was stored at 4°C.

Adsorbed plasma with bentonite: Bentonite (Kanto Kagaku Co., Ltd., Tokyo) was added to pooled citrated plasma (50 mg/ml plasma) and stirred with a plastic rod for 20 min at 25°C. After centrifugation at 3,000 rpm for 5 min, the supernatant was stored at 4°C.

Stored citrated plasma: Pooled citrated plasma was incubated at 37°C for 24 h and stored at 4°C.

Procedure for Determination of the Clotting Time

The procedures for the determination of clotting time were the same as those described in a previous paper¹⁾ except that the manner of moving the test tube was modified as follows.

In the determination of recalcification time (RT), prothrombin time (PT) and partial thrombo-

* Department of Fisheries, Faculty of Agriculture, Miyazaki University, Kumano, Miyazaki 889-21, Japan
(川津浩嗣, 佐藤 誠: 宮崎大学農学部水産増殖学科).

plastin time (PTT), a rough clotting time was determined using the first tube. For the first tube, clot formation was checked at 60-second intervals by moving the tube for several seconds. For example, when clot formation was observed after 3 min, the clotting time was recorded as 2+ min. When a more exact clotting time had to be obtained, the second tube was moved continuously after 2 min and the exact clotting time was recorded. The exact clotting time was found to be between 2 and 3 min. Duplicate readings were obtained from each sample and the mean clotting time was recorded.

Test Tubes

Three kinds of test tubes were used for the determination of the clotting time: glass tubes (10×90 mm), silicone treated glass tubes (10×90 mm) and polystyrene tubes (10×72 mm). For the preparation of the silicone treated tubes, KC 88 silicone (Shin-etsu Chemical Co., Ltd.) was used.

Procedure for Detection of Contact Effect

Glass beads and ellagic acid were used for the detection of the contact effect. Glass beads (75–150 μ m) were obtained from Sigma Chemical Co., Ltd., St. Louis, Mo., USA, and ellagic acid from Aldrich Chemical Co., Ltd., Milwaukee, Wis., USA. Glass contact was performed by mixing glass beads with plasma in a plastic tube and by gently rotating the tube at 25°C for 30 min. Ellagic acid was dissolved with 0.85% NaCl at varied concentrations. Equal volumes of ellagic acid solution and plasma were mixed in a plastic tube at room temperature. Clotting times of the original and contacted plasma were determined.

Results

Comparison of RT Obtained from Glass, Silicone Treated Glass and Polystyrene Tubes

The determination of RT was performed using three kinds of test tubes: glass, silicone treated glass and polystyrene tubes. Clot formation was observed only in the glass tubes, and no clot was formed in either the silicone treated glass or polystyrene tubes within 20 min (Table 1). This fact suggests that the citrated plasma contains a certain coagulation factor which is converted to an activated form by contact with glass and that the activated form triggers clot formation.

In order to confirm the above supposition, RT

Table 1. Comparison of RT obtained from 8 plasma samples using three kinds of test tubes

Plasma	RT (seconds)		
	Glass tubes	Silicone treated glass tubes	Polystyrene tubes
1	43	no clotting*	no clotting
2	56	no clotting	no clotting
3	107	no clotting	no clotting
4	107	no clotting	no clotting
5	124	no clotting	no clotting
6	130	no clotting	no clotting
7	140	no clotting	no clotting
8	177	no clotting	no clotting
mean $\pm$ SD	111 $\pm$ 41		

* no clotting within 20 min.

Table 2. RT of the contact plasma with glass beads

Amount of glass beads added (mg/ml plasma)	RT (min)	
	Plasma No. 1	Plasma No. 2
0	no clotting	no clotting
50	no clotting	no clotting
100	no clotting	no clotting
500	6+	6+
750	4+	4+
1,000	3+	3+
1,500	—	3+

Table 3. RT of the contact plasma with ellagic acid

Concentration of ellagic acid	RT (min)		
	Plasma No. 1	Plasma No. 2	Plasma No. 3
10 ⁻³ M	2+	2+	2+
10 ⁻⁴ M	3+	11+	no clotting
10 ⁻⁵ M	4+	no clotting	no clotting
10 ⁻⁶ M	no clotting	no clotting	no clotting

was determined for the plasma incubated with varying amounts of glass beads. Clot formation was observed when more than 500 mg of glass beads was added per ml of plasma and RT was shortened when the amount of added glass beads was increased (Table 2).

In the determination of RT for the mixtures of ellagic acid and plasma, clot formation was observed in the mixtures with 10⁻³–10⁻⁵ M ellagic acid. At concentrations of less than 10⁻⁶ M ellagic acid, no clots were formed (Table 3).

Table 4. Comparison of APTT obtained from 5 plasma samples using three kinds of test tubes

Plasma	APTT (seconds)		
	Glass tubes	Silicone treated glass tubes	Polystyrene tubes
1	4.0	3.8	4.3
2	4.8	4.9	5.4
3	5.2	5.4	6.1
4	5.3	5.6	5.5
5	5.3	5.9	5.9
mean $\pm$ SD	4.9 $\pm$ 0.5	5.1 $\pm$ 0.7	5.4 $\pm$ 0.6

Table 5. Comparison of PTT obtained from 10 plasma samples using three kinds of test tubes

Plasma	PTT (seconds)		
	Glass tubes	Silicone treated glass tubes	Polystyrene tubes
1	14	144	144
2	16	180	172
3	18	109	107
4	18	114	114
5	20	170	170
6	20	175	177
7	22	178	173
8	23	142	123
9	24	171	168
10	28	167	159
mean $\pm$ SD	20.3 $\pm$ 3.9	155 $\pm$ 25	151 $\pm$ 25

Comparison of APTT Obtained from Glass, Silicone Treated Glass and Polystyrene Tubes

No significant differences were observed between the values of APTT obtained from glass, silicone treated glass and polystyrene tubes (Table 4). This result indicates that the effect of glass contact was negligible compared with the effect of ellagic acid in the determination of APTT.

Comparison of PTT Obtained from Glass, Silicone Treated Glass and Polystyrene Tubes

Significant differences were observed between the values of PTT obtained from glass, silicone treated glass and polystyrene tubes (Table 5). The clotting time obtained from silicone treated glass and polystyrene tubes was several times longer than that obtained from glass tubes. Shortening of the clotting time in the glass tube can be attributed to the activation of a certain coagulation factor induced by glass contact. Furthermore, clot formation in silicone treated glass and polystyrene tubes suggests that cephalin also plays a

Table 6. RT and PTT simultaneously obtained from the same plasma sample using glass and polystyrene tubes

Plasma	Polystyrene tubes		Glass tubes	
	RT	PTT	RT	PTT
1	no clotting* ¹	99* ²	56* ²	26* ²
2	no clotting	162	84	25
3	no clotting	155	143	26
4	no clotting	210	147	27
5	no clotting	191	155	34
mean $\pm$ SD	—	163 $\pm$ 38	117 $\pm$ 40	28 $\pm$ 3

*¹ no clotting within 20 min.

*² seconds.

Table 7. Comparison of PT obtained from 12 plasma samples using three kinds of test tubes

Plasma	PT (seconds)		
	Glass tubes	Silicone treated glass tubes	Polystyrene tubes
1	36	210	238
2	41	304	309
3	43	82	83
4	43	314	323
5	44	259	332
6	45	130	130
7	47	117	121
8	49	473	436
9	57	142	132
10	64	171	231
11	68	183	191
12	69	238	252
mean $\pm$ SD	50.5 $\pm$ 10.7	218.6 $\pm$ 103.9	231.5 $\pm$ 101.2

role as a trigger in the intrinsic coagulation system.

In order to estimate the interaction of glass contact and addition of a partial thromboplastin (PTT reagent), RT and PTT were simultaneously determined for each plasma sample using glass and polystyrene tubes. The interaction effect was judged to be highly significant (Table 6).

Comparison of PT Obtained from Glass, Silicone Treated Glass and Polystyrene Tubes

Significant differences were also observed between the values of PT obtained from glass, silicone treated glass and polystyrene tubes (Table 7). The clotting times obtained from silicone treated glass and polystyrene tubes were about 4 times longer than that obtained from glass tubes. Shortening of the clotting time in the glass tubes can be attributed to the activation of a certain

Table 8. Prothrombin time (PT) of plasma mixtures

	PT (min) before mixing	PT (min) after mixing with		
		BaSO ₄ adsorbed plasma	Stored serum	Bentonite adsorbed plasma
Citrated plasma incubated at 37°C for 24 h	no clotting* ¹	no clotting	6+* ²	no clotting
BaSO ₄ adsorbed plasma	no clotting	—	3+* ³	8+* ²
Stored serum	no clotting	3+	—	no clotting
Bentonite adsorbed plasma	no clotting	8+	no clotting	—

*¹ No clotting within 20 min.*² Fibrin web appeared.*³ Fibrin clot formed.

coagulation factor induced by glass contact.

PT of the Plasma Mixtures

The PT was determined in mixtures of the following plasma and serum samples: stored citrated plasma, adsorbed plasma with BaSO₄, adsorbed plasma with bentonite, and stored serum. To prepare the plasma mixtures, equal volumes of each plasma or serum sample were mixed. Polystyrene tubes were used for measuring the clotting time.

The results are summarized in Table 8. The original plasma and serum samples did not clot within 20 min. Fibrin clot formation was observed in the mixture of stored serum and adsorbed plasma with BaSO₄, and appearance of a fibrin web was observed in the following two mixtures: stored citrated plasma+stored serum, and adsorbed plasma with BaSO₄+adsorbed plasma with bentonite. These results approximately corresponded with those described in a previous paper.¹⁾

Discussions

The citrated plasma did not clot in silicone treated glass and polystyrene tubes by the addition of calcium chloride alone (Table 1), but clotted after being contacted with glass beads or ellagic acid (Tables 2 and 3). These results suggest that plasma contains a XII-like coagulation factor, which is converted to the activated form by contact with glass or ellagic acid, the activated form functioning as a trigger in the intrinsic coagulation system.

The citrated plasma clotted in silicone treated glass and polystyrene tubes, when recalcified in the presence of an activated partial thromboplastin (APTT reagent), or a partial thromboplastin (PTT reagent). No noticeable difference was observed in APTT between the values obtained from the glass tubes and silicone treated glass or polystyrene

tubes (Table 4), while remarkable differences were observed in PTT (Table 5). These results indicate that shortening of the clotting time in PTT is attributable to the effect of glass contact, while in APTT the effect of glass contact was masked due to the presence of ellagic acid. Furthermore, clot formation in silicone treated glass or polystyrene tubes in the determination of PTT suggests that cephalin is capable of functioning as a trigger in the intrinsic coagulation system without aid of the XII-like factor. This is also confirmed in the experiment shown in Table 6.

Striking differences were also observed in PT between the values obtained from glass tubes and silicone treated glass or polystyrene tubes (Table 7). The shortening of the clotting time is also attributable to the effect of glass contact. The activated form of factor XII, Factor XI and kallikrein have been reported to be capable of converting factor VII to activated form in mammalian blood.^{2,3)} This means that activation of factor XII in the intrinsic system triggers the functioning of the extrinsic system and both systems work coincidentally. The existence of such a pathway in fish blood still remains to be studied in the future.

The data shown in Table 8 reconfirmed the previous finding that coagulation factors in carp blood can be classified into two categories: the serum factors and the barium sulphate adsorbed factors.

This work was supported by a Grant-in-Aid for Scientific Research of the Ministry of Education.

References

- 1) H. Kawatsu: *Nippon Suisan Gakkaishi*, **53**, 591-595 (1986).
- 2) W. Kisiel, K. Fujikawa, and E. W. Davie: *Biochemistry*, **16**, 4189-4194 (1977).
- 3) E. W. Davie, K. Fujikawa, K. Kurachi, and W. Kisiel: *Adv. Enzymol.*, **48**, 277-318 (1979).