의학석사 학위논문

Evaluation of residual samples as substitute for normal donor platelets in functional tests for heparin-induced thrombocytopenia

헤파린 유도 혈소판감소증 진단을 위한 기능검사에서 사용되는 정상 기증 혈소판의 대체제로서 잔여 검체 평가

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유준상

Evaluation of residual samples as substitute for normal donor platelets in functional tests for heparin-induced thrombocytopenia

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이 논문을 의학석사 학위 논문으로 제출함

2022년 08월

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유준상

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Abstract

Heparin induced thrombocytopenia (HIT) is a prothrombotic disease caused by platelet activating antibody against platelet factor 4 (PF4) and heparin complex. Widely available laboratory tests for HIT, such as immunoassays to detect antibody against PF4/heparin complex lack specificity especially in optical density of 0.4-2.0. Functional assays, such as serotonin release assay, heparin-induced platelet aggregation test (PAT), and heparin-induced platelet activating (HIPA) assay are tests with specificity, but they are not widely used due to limitations including a need of platelets from normal healthy donors, variable reactivity of platelets, and relatively short time of availability of platelets. For these limitations, testing with selected donors whose platelets are known to react strongly to functional test or 4 random donors to interpret positive when platelets from more than 2 donors show react. To overcome these limitations, we evaluate residual samples as substitute for platelets from normal donors in functional tests for HIT.

We evaluated PAT and modified HIPA assay with a PF4/heparin antibody positive plasma and platelets from residual samples from health screening and promotion center. Residual samples were re-suspended and centrifuged at 200g for 10 minutes to make platelet rich

plasma (PRP). PRPs were collected into one bottle and tested with positive sample and heparin concentration of 0, 1 and 100 IU/mL in PAT. Washed platelets (WPs) were made from pooled PRPs by centrifuge at 1000g for 8 minutes twice with eliminating supernatant and resuspended with phosphate-buffered isotonic saline. Then, modified HIPA assay was done with heparin concentration of 0, 0.2 and 100 IU/mL with light transmission aggregometry. We tested 10 PAT and 26 modified HIPA test and positive rate of 20% and 92%, respectively. Considering it was tested with the PF4/heparin antibody positive plasma, positive rate for PAT was low to perform, but modified HIPA assay showed high positive rate. Since it is difficult to obtain donor platelets in functional assays, they are not widely used. Platelets obtained from residual samples may be an alternative in the future, so further studies are needed.

Keyword: heparin, thrombocytopenia, platelet function test, platelet-rich plasma, platelet aggregation, platelet factor 4

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Background

Heparin induced thrombocytopenia (HIT) is caused by platelet activating antibody against platelet factor 4 (PF4)/heparin complex. Pathogenesis of HIT is explained by this theory [1]. PF4 is known to exist as an equilibrium among tetramers, dimers and monomers in plasma or whole blood. Tetramers are thought to be transient and switch between 'open-closed' and 'closed-open' conformations. When heparin binds to 'closed' end of PF4 tetramer, it stabilizes the PF4 tetramers, so 'open' end of the tetramer is exposed. Stabilization of the PF4 tetramers enhances binding of pathogenic antibodies to 'open' end, forming IgG/PF4/heparin immune complex that activate platelet through FcγRIIAs and expression of tissue factor, which leads to thrombin-dependent transactivation of platelets and endothelial cells.

HIT typically occurs 4-10 days after heparin exposure. Thrombocytopenia occurs and platelet count usually declines by at least 50% over the next 2-3 days. In HIT, platelet count rarely falls below 10 x 10^9/L. Unlike other thrombocytopenic diseases, HIT is prothrombotic disease that venous thrombosis of the large vessels of the lower limbs and pulmonary embolism are the most frequent complications, but if can affect the cerebral sinus or splanchnic veins. Overt disseminated intravascular coagulation also occur in 5-10%. Despite

optimal management, disease morbidity and mortality remain high, so the diagnosis and early management of HIT is important.

While using heparin, thrombocytopenia with thrombosis or other sequelae develop, clinical suspicion of HIT can be made. Combining these together, the 4Ts scoring system is available to diagnosis HIT [2]. 4Ts stand for thrombocytopenia, timing of platelet count fall, thrombosis and other causes of thrombocytopenia. The 4Ts score is the sum of the values for each of the 4 categories. Score of 1-3, 4-5 and 6-8 are considered to correspond to a low, intermediate, and high probability of HIT, respectively.

Widely available laboratory tests for HIT, such as enzyme immunoassays (EIAs) have high sensitivity, but low specificity especially in optical density (OD) value of between 0.4 and 2.0 [3]. Functional platelet activation assays have high specificity. Examples of functional assays for HIT are heparin-induced platelet aggregation test (PAT), heparin-induced platelet activation (HIPA) assay, serotonin-release assay, and PF4-dependent P-selectin expression assay. Even with high specificity, functional assays are not widely used because of limitations including a need of platelet from normal healthy donors, variable reactivity of platelets, and relatively short time of availability of platelets. For these limitations, testing with selected donors whose platelets are known to react strongly to functional test or 4

random donors to interpret positive when platelets from more than 2 donors show react. Even with these approaches, fresh platelets from healthy donors are needed every test. In this study, to overcome limitations of functional assays, we studied PAT and HIPA assay using platelets from residual samples.

Material and method

1. Sample collection and processing

Residual samples of coagulation tests requested from the health screening and promotion center, were collected. Residual samples were collected in citrate tube in (3.2% sodium citrate). In addition to visually hemolytic, icteric, and lipidemic samples, samples from persons younger than 20 years and those over 60 years of age were excluded. Collected samples were resuspended and centrifuged at 200g for 10 minutes to make PRPs. Then, 4 to 8 PRPs were pooled into one tube. For HIPA assay, PRP was centrifuged at 1000g for 8 minute and platelet pellet was re-suspended in phosphate-buffered isotonic saline for two times to make washed platelet (WP). HIT positive plasma was from a patient confirmed as high OD value with HemosIL AcuStar HIT-IgG (PF4/H) (Werfen, MA, USA).

2. PAT test

We performed the PAT test as described with a few modifications [4]. We used residual samples for PRP rather than blood of normal donors. Each PRPs were from 4-8 different residual samples. Aggregation was observed by light transmission aggregometer (Chrono-log, PA, USA). We incubated 350uL of PRP, 50uL of HIT positive plasma, and 10uL of heparin.

Heparin concentration of 0, 1.0, 100 IU/mL were tested. The other one well was tested with platelet poor plasma (PPP) from same residual samples rather than HIT positive plasma because of possible PF4/heparin antibody (Table 1A). 4 wells were with magnetic stirring bar tested for 20 minutes at 37°C.

3. Modified HIPA assay

We performed HIPA assay as previously described with a few modifications [4]. We used pooled platelets from residual samples for WPs. Rather than observing visually every 5 minutes for 45 minutes, we assessed aggregation by light transmission aggregometer for 20 minutes. We incubated 350uL of WP, 50uL of HIT positive plasma, and 10uL of heparin. Heparin concentration of 0, 1.0, 100 IU/mL were tested (Table 1B). Positive result was judged as same as PAT.

4. Platelet count and ABO blood type

Platelet counts of residual samples were obtained from existing data in medical record. ABO blood types of residual samples were obtained from medical record, if available. Platelet counts of pooled PRPs and pooled WPs were obtained by testing with automated hematology analyzer XN-2000 (Sysmex, Seoul, Korea).

5. Positive result criteria

For both PAT and modified HIPA assay, platelet aggregation of ≥50 percent in the presence of low concentration heparin, no aggregation in the absence of heparin and inhibited aggregation more than 50 percent in the presence of high concentration heparin than lower concentration was judged as a positive result. Inconclusive results were made if aggregation was observed in tube without heparin and/or not inhibited by high concentration heparin. No aggregation with low concentration heparin was considered negative result.

6. Reactivity check

We checked reactivity of platelet from residual samples in PAT and modified HIPA assay with same HIT positive plasma used in PAT and modified HIPA. For PAT test, we tested platelets from residual samples and low concentration heparin (1.0 IU/mL) with HIT positive plasma and PPP from same residual samples. PPP was tested for potential antibody against PF4/heparin complex in residual samples. For modified HIPA assay, platelets from residual samples were tested with low concentration heparin (0.2 IU/mL) and HIT positive plasma. Since modified HIPA assay is using washed platelets, PPP was not tested.

Table 1A. Test method of heparin-induced platelet aggregation test

	Well 1	**Well 2**	**Well 3**	**Well 4**
Volume 350㎕	PRP	PRP	PRP	PRP
Plasma 50㎕	PPP	HIT positive plasma	HIT positive plasma	HIT positive plasma
Heparin 10㎕	1.0 IU/mL	0 IU/mL	1.0 IU/mL	100 IU/mL

Table 2B. Test method of modified heparin-induced platelet activation assay

	Well 1	**Well 2**	**Well 3**
Volume 350㎕	WP	WP	WP
Plasma 50㎕	HIT positive plasma	HIT positive plasma	HIT positive plasma
Heparin 10㎕	0 IU/mL	0.2 IU/mL	100 IU/mL

Abbreviation: PRP, platelet rich plasma; WP, washed platelet; PPP, platelet poor plasma; HIT, heparin-induced thrombocytopenia

Result

We collected 47 residual samples for PAT test and 118 residual samples for modified HIPA assay. 47 residual samples for PAT were pooled into 10 PRPs, and 118 residual samples for modified HIPA assay were pooled into 26 WPs. Data for platelet count of initial CBC data, PRP, washed platelet is summarized in table 2. Initial CBC data was missed in 74 residual samples and platelet count was missed in 18 pooled PRPs and 2 pooled WPs. Median platelet count was 249 x $10^3/\mu\ell$ for initial CBC data, 309.5 x $10^3/\mu\ell$ for PRPs and 319 x $10^3/\mu\ell$ for washed platelets. It was observed that platelet count was not decreased significantly and adequate for the tests.

We tested total 10 PAT and 26 modified HIPA assays. Pooled PRPs from 4 to 5 residual samples were used in PAT, and pooled washed platelets from 4 to 8 residual samples were used in modified HIPA test. Residual samples of same ABO type were pooled in 9 PAT and 8 modified HIPA assay. Residual samples of different ABO types were pooled in 1 PAT and 3 modified HIPA assay. Patients with no data of ABO type were pooled randomly in 16 modified HIPA assay.

Summarized results of PAT test are presented in Table 3. Positive, inconclusive and negative rate were 20%, 30% and 50% respectively. Aggregation in high heparin

concentration was not inhibited in 3 inconclusive results. Median value of maximum aggregation percent with 1.0 IU/mL heparin concentration were 86.5% in positive results, 69.5% in inconclusive results, and 34.5% in negative results. One positive sample was from 4 residual samples of O blood type and the other was from 4 residual samples of A blood type (Table 5).

24 out of 26 tests modified HIPA assay showed positive result and 2 tests showed inconclusive result (Table 4). Two inconclusive test showed nonspecific aggregation without heparin (Figure 1B). Median value of maximum aggregation percent with 0.2 IU/mL heparin concentration were 85.5% in positive results and 51.5% in inconclusive results. 2 out of 2 modified HIPA assays with mixed ABO blood types showed positive results. 14 out of 15 randomly mixed samples with no ABO blood type data showed positive result. All randomly mixed samples were pooled from 4 residual samples. 7 out of 8 tests with same ABO blood types were positive (Table 6).

Total Reactivity check for 21 were done to check reactivity of PAT. Each test was done with a pair of two samples with same blood types. 4 tests, 1 test and 16 tests showed positive, intermediate and negative results, respectively. Positive rate in reactivity test was 19% (Table 7). For modified HIPA assay, 30 out of 35 tests (86%) showed positive results, and 5 showed

negative result (Figure 2). 9 tests were done with a pair of two plasma with same blood type, and 26 tests were done with a plasma. If tests with one plasma were counted, positive rate was 81% (21/26) (Table 8).

Table 2. ABO blood type and platelet count of initial CBC data, platelet rich plasma and washed platelets

ABO blood type	Initial platelet count	PRP	ABO blood type	Initial platelet count	PRP	Washed platelet
B+	230	388	O+	335	341	320
	243			288		
	251			158		
	284			175		
	197			258		
O+	291	358	O+	237	246	122
	247			248		
	168			206		
	195			198		
B+	234	252		267		
	255		B+	297	252	121
	260			211		
	307			190		
A+	291	329		213		
	306			217		
	277		B+	210	307	318
	212			243		
B+	316	240		206		
	230			327		
	218			196		
	245		A+	272	312	343
	423			241		
O+	202	234		278		
	266			252		
A+	262			293		
	213		O+	224	305	469
	231			261		
O+	277	288		202		
	273			249		
	240			253		
	249		A+	264	336	114
	224			234		
O+	315	377		236		
	282			274		
	386		O+	328		
	174		B+	277	357	419
AB+	193	285		299		
A+	276			239		
	238			204		
	251		A+	228		
	201			290		
				282		
			AB+	325		
			A+	330	340	244
			B+	202		
			O+	278		
			AB+	308		
				265		
				189		

* Platelet count of washed platelet that didn't have data of initial CBC and PRP are not shown.

Abbreviation: CBC, complete blood count; PRP, platelet rich plasma

Table 3. Results of heparin-induced platelet aggregation test

Result	Number	Median of maximum aggregation % with heparin 1 IU/mL (95% confidence interval)
Positive	2 (20%)	86.5 (29.3-143.7)
Inconclusive	3 (30%)	69.5 (-51.2-190.2)
Negative	5 (50%)	34.5 (27.3-41.7)

Table 4. Results of modified heparin-induced platelet activating assay

Result	Number	Median of maximum aggregation % with heparin 0.2 IU/mL (95% confidence interval)
Positive	24 (92%)	85.5% (81.3-89.7%)
Inconclusive	2 (8%)	51.5%

Figure 1. Aggregation curves of positive result (A) and inconclusive result (B) of modified heparin-induced platelet activating assay using pooled platelets from residual samples

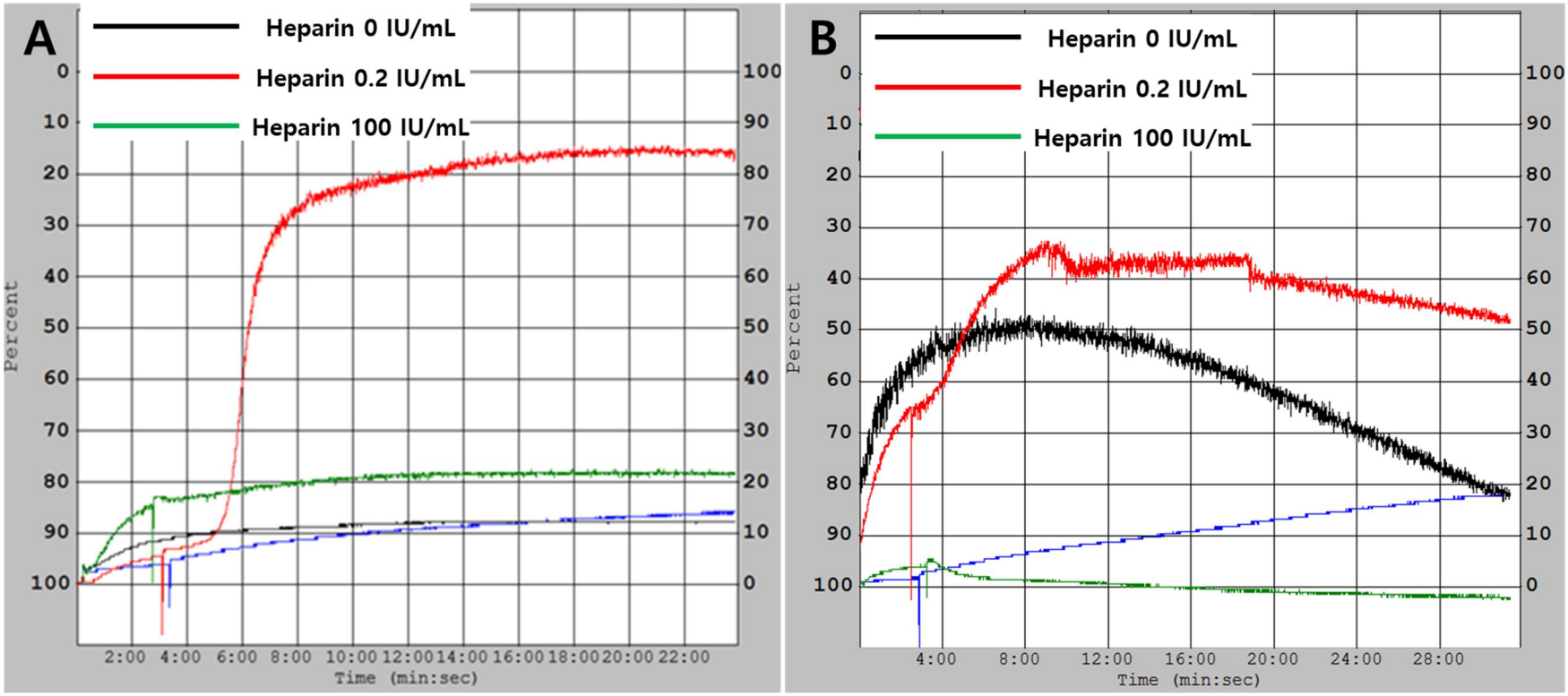


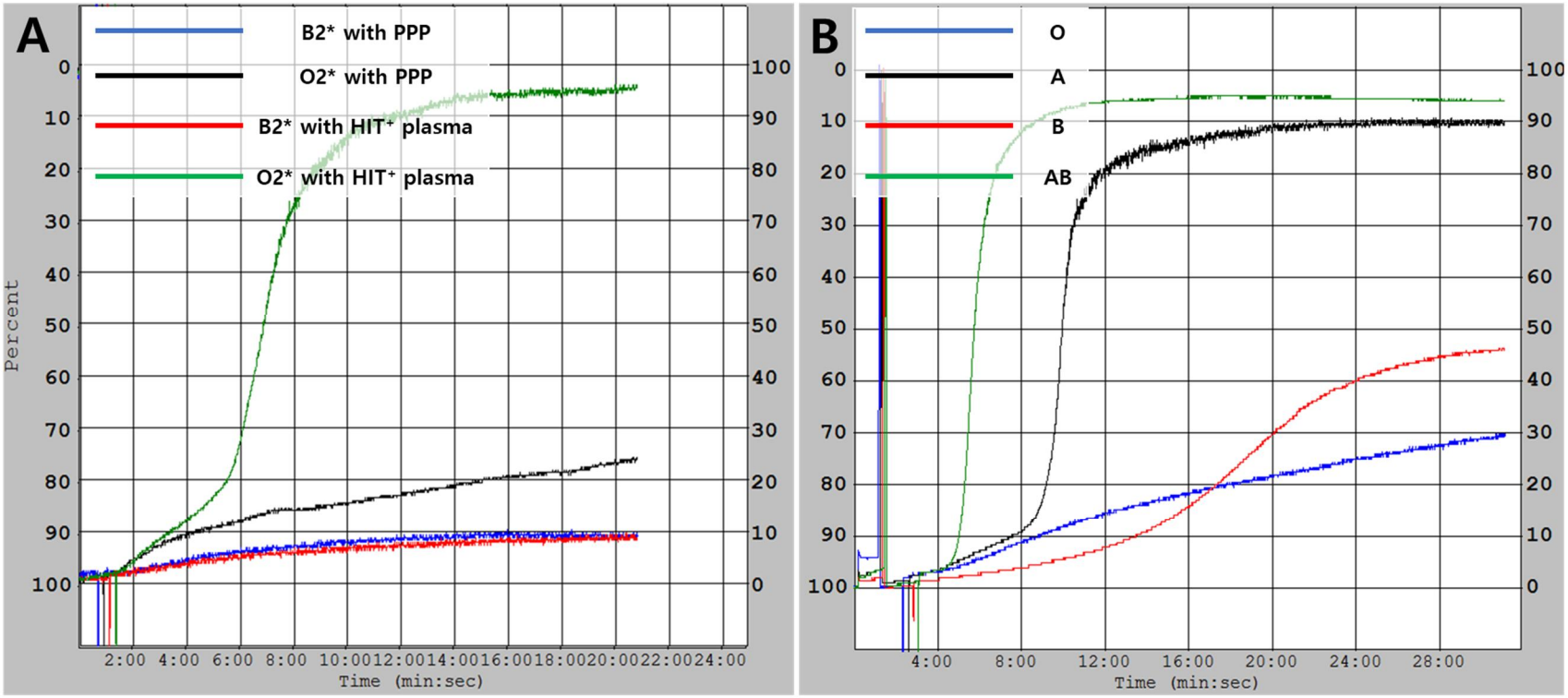
Table 5. ABO blood type, platelet count and result of heparin-induced platelet aggregation test (PAT)

ABO blood type	Initial platelet count	PRP platelet count	PAT result
B+	230 243 251 284 197	388	Inconclusive
O+	291 247 168 195	358	Positive
B+	234 255 260 307	252	Inconclusive
A+	291 306 277 212	329	Positive
B+	316 230 218 245 423	240	Negative
O+	202 266	234	Negative
A+	262 213 231		
O+	277 273 240 249 224	288	Inconclusive
O+	315 282 386 174	377	Negative
AB+	193	285	Negative
A+	276 238 251 201		

Table 6. ABO blood type, platelet count and result of heparin-induced platelet activation (HIPA) assay

ABO blood type	Initial platelet count	WP platelet count	Modified HIPA assay result
O+	335 288 158 175 258	320	Positive
O+	237 248 206 198 267	122	Positive
B+	297 211 190 213 217	121	Positive
B+	210 243 206 327 196	318	Inconclusive
A+	272 241 278 252 293	343	Positive
O+	224 261 202 249 253	469	Positive
A+	264 234 236 274	114	Positive
O+	328		
B+	277 299 239 204	419	Positive
A+	228 290 282		
AB+	325		
A+	330	244	Positive
B+	202		
O+	278		
AB+	308 265 189		

Figure 2. Aggregation curves of reactivity test of heparin-induced platelet aggregation test (PAT) (A) and modified heparin-induced platelet activation (HIPA) assay (B)



Reactivity test of heparin-induced platelet aggregation test (A) showed negative result in B2 and positive in O2. In modified heparin-induced platelet activation assay (B), positive results are shown in A and AB; negative results are shown in O and B.

* Pooling of two platelet rich plasmas with same ABO blood type from residual samples

Abbreviation: PPP, platelet poor plasma; HIT^{+} plasma, heparin induced thrombocytopenia positive plasma

Table 7. Reactivity test; ABO blood type and result of heparin-induced platelet aggregation test

혈액형	Reactivity
A2	negative
O2	negative
B2	negative
O2	positive
O2	negative
A2	negative
A2	negative
B2	inconclusive
A2	negative
B2	negative
AB2	negative
O2	negative
A2	positive
B2	positive
O2	positive
A2	negative
B2	negative
AB2	negative
O2	negative
A2	negative
O2	negative

Table 8. Reactivity test; ABO blood type and result of modified heparin induced platelet activation assay

혈액형	Reactivity
A2	positive
A2	positive
A2	positive
A2	positive
A2	positive
B2	positive
AB2	positive
O2	positive
B2	positive
O	negative
A	positive
B	negative
AB	positive
A	positive
A	positive
O	positive
O	positive
B	positive
B	positive
A	negative
O	positive
AB	positive
O	positive
O	negative
A	positive
A	positive
A	positive
B	negative
B	positive
O	positive
A	positive
B	positive
O	positive
O	positive
O	positive

Discussion

This study was aimed to overcome limitation of functional tests of HIT using residual samples. Platelet count was not significantly decreased when using residual samples (Table 2). There are two washed platelets that had low platelet count with 121 and 114 x $10^3/\mu\ell$, but both were positive in modified HIPA test. A method of more consistently maintaining platelet counts is needed, but it does not appear to be a major problem when using residual samples.

PAT showed positive rate of 20% (2/10). Indirectly comparing with sensitivity of PAT from other study which is 39% to 81% [5], positive rate of PAT using residual sample is low to perform routinely. However, modified HIPA showed potential with positive rate of 92% (24/26). Since we used only HIT positive plasma with high OD value, further studies with HIT negative and HIT positive plasma with OD value of 0.4-2.0 should be needed to evaluate performance of functional tests HIT using residual samples.

Apyrase was not used during platelet washing in this study. Apyrase can be used when preparing washed platelet to prevent the accumulation of ADP and becoming refractory to ADP-mediated platelet activation [6]. Other studies of HIPA also used apyrase [4, 7]. Further studies are needed using apyrase during platelet washing.

In reactivity check, positive rate was 19% for PAT and 85% for modified HIPA assay. Positive rate in PAT were too low to be performed. If we use 4 pooled PRPs from residual samples, about 99.95% will have at least one reactive PRP when calculating with 85% of positive rate. In previous study, platelets from normal donors showed variable reactivity with HIT positive sera [8]. Our study only used HIT positive plasma with high OD value, data of reactivity rate with plasma with OD value of 0.4-2.0 is needed since widely available laboratory tests such as EIA show low specificity in this OD value.

It is known that tests using donor platelets should be performed within 4 hours after blood collection [9]. Data is not shown in this study, but we tested 3 pooled PRPs from residual samples with collagen within 4 hours, 6 hours, 12 hours, and 24 hours after blood collection, respectively. All 3 PRPs showed positive test until 12 hours. 2 PRPs were positive until 24 hours. More studies are needed, but this small data suggest that usage of PRPs more than 4 hours can be considered.

Absence of A and B blood type antigen in platelet surface and suggestion that ABO status is inconsequential in previous study [10], were the reasons we pooled different ABO types. We mixed different ABO types in two pooled PRPs in PAT and three washed platelets in modified HIPA assay. Also, we tested HIPA assay with 15 washed platelets pooled from 4

residual samples each, which ABO types were not available. Two pooled PRPs were negative in PAT, but 3 ABO type known washed 15 platelets and randomly pooled washed platelet showed 94% of positive rate (17/18). These data suggest that different ABO types could be pooled together in platelet functional tests.

Functional tests for HIT are useful to confirm the diagnosis of HIT especially in low OD values in widely available laboratory tests including chemiluminescence immunoassay. Also, recent study suggested that COVID-19 patients often present strong reactivity to immunoassays that detect antibody against PF4/heparin complex, but negative result in functional tests [11]. Therefore, functional tests are more necessary for an accurate diagnosis of HIT. Unfortunately, functional tests require platelets from a healthy donor. In HIPA test, platelets from 4 donors are used in each test [4]. Serotonin release assay use selected platelet donors known to react well with HIT antibodies [10]. If residual samples can be used as an alternative to donor platelets, more laboratories can easily test functional assays. Our study suggests possibility of using residual samples in functional assays, especially that use washed platelets. Further studies are needed to establish test method and to evaluate test performance of functional assays.

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Korean abstract

헤파린 유도 혈소판 감소증은 platelet factor 4 (PF4)와 헤파린 복합체에 대한 혈소판 활성화 항체에 의해 유발되는 혈전성 질환이다. PF4/헤파린 복합체에 대한 항체를 검출하기 위해 널리 사용되는 헤파린 유도 혈소판 감소증에 대한 검사인 면역측정법 등은 0.4에서 2.0의 optical density (OD) 값에서 특이도가 낮다. Serotonin release assay, heparin-induced platelet aggregation test (PAT), heparin-induced platelet activation (HIPA) 검사 등의 기능 검사는 특이도가 높은 검사이지만 정상인의 혈소판을 필요로 하는 점, 혈소판의 반응도의 다양성, 상대적으로 짧은 혈소판의 이용가능한 시간 등의 한계점으로 널리 활용되지 못하고 있다. 이러한 계점들을 극복하기 위해 혈소판이 기능 검사에서 강하게 반응하는 것으로 알려진 기증자를 사용하거나 4명의 무작위 기증자의 혈소판 중 2명 이상의 기증자의 혈소판이 반응을 보일 때 양성으로 해석하는 방법들이 현재 이용되고 있다. 이번 연구에서는 이러한 한계점들을 극복하기 위해 헤파린 유도 혈소판 감소증에 대한 기능 검사에서 정상인 기증자의 혈소판을 대체하기 위해 잔여 검체를 사용했습니다.

건강증진센터에 의뢰된 검체들로부터 얻은 혈소판과 PF4/헤파린 항체 양성 혈장을 이용해 PAT와 modified HIPA 검사를 평가했습니다. 잔여 검체를 재부유 시킨 후, 10분동안 200g로 원심 분리하여 혈소판 풍부 혈장을 만들었습니다. PAT는 혈소판 풍부 혈장을 한 튜브에 모은 후 각각 0.1, 1, 100IU/mL 농도의 헤파린 용액과 양성 검체와 반응시켜 검사

했습니다. 세척 혈소판은 모은 PRP를 8분동안 1000g로 원심분리하여 상층액을 버린 후 phosphate-buffered saline를 넣고 다시 재부유 시켜서 만들었습니다. 그 후, 얻은 세척 혈소판을 광투과 혈소판응집검사를 이용하여 0, 0.2, 100IU/mL 농도의 헤파린 용액과 양성 검체와 반응시켜 검사했습니다. 10개의 PAT 검사와 26개의 modified HIPA 검사를 시행했고, 각각 20%, 92%의 양성률을 보였습니다. PF4/heparin 항체 양성 혈장으로 검사한 것을 감안하면 PAT 양성률은 낮았지만 modified HIPA 검사에서는 높은 양성률을 보였습니다. 기능 검사는 정상인 기증자로부터 혈소판을 확보하기 어렵기 때문에 널리 사용되고 있지 못하고 있다, 잔여 검체에서 얻은 혈소판이 대안이 될 수도 있기에 이에 대한 연구가 더 필요하다.