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Effects of Spectral Interfering Substances on Light Transmission Platelet Aggregation Using Infrared Based Aggregometer

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ABSTRACT

Aim: This study aims to investigate the nature and extent of hemolyzed, icteric, or lipemic (HIL) interference on platelet aggregation (PA) using the TA-8V aggregometer (Diagnostica Stago, Asnière sur Seine) equipped with a near infrared light source outside the typical absorbance range of HIL.

Methods: Platelet-Rich-Plasma (PRP) samples were spiked with substances mimicking HIL interference: red blood cell hemolysate (RBCH; 0.3–20 g/L of hemoglobin), bilirubin (15–400 mg/L), and a fat emulsion (Intralipid 20%: 0.5–3 g/L). Maximal intensity (MaxInt) and velocity (Vel) were recorded in the basal state and in response to ADP 5 µmol/L and collagen 2 µg/mL. RBCH solution was treated with apyrase 0.1 U/mL.

Results: Spontaneous aggregation appeared above 0.6 g/L RBCH and significantly intensified with increased RBCH concentrations. The addition of apyrase to RBCH prevented spontaneous aggregation regardless of the RBCH concentration and led to reduced interindividual variability. In response to ADP and collagen, MaxInt and Vel significantly decreased as apyrase-treated RBCH concentrations increased. MaxInt and Vel in response to ADP or collagen were not affected by increasing concentrations of bilirubin. The presence of lipids significantly increases MaxInt in response to ADP or collagen starting at 0.5 g/L.

Conclusion: Our findings suggest that PA testing using the TA-8V instrument is not significantly impacted by icterus and hyperlipidemia within the specified ranges in healthy individuals. However, it is crucial to reject grossly hemolysed samples (exceeding 0.6 g/L) to avoid interference with ADP released from red blood cells. Further research is needed to confirm these results in patients with platelet dysfunction.

1 | Introduction

Light transmission aggregometry (LTA), first described in the early sixties [1–3], is commonly used for assessing platelet function. LTA measures the turbidity of platelet-rich plasma (PRP) at a specific wavelength and observes platelet aggregation as a decrease in turbidity over time on a curve. LTA remains the gold standard technique for the diagnosis of acquired or inherited platelet function disorders. Platelet-poor plasma (PPP) is considered the reference point for 100% transmission. When using the turbid PRP, the maximum light transmission is absorbed and thus considered 0% transmission. The addition of platelet aggregation activators initiates platelet aggregation in the sample, leading to a decrease in turbidity and an increase in light

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transmission. Typically, a photosensor detects the level of light passing through the solution from a light source. Hemolysis, icterus, or lipemia (HIL) are common occurrences in routine clinical laboratory practice and can cause biological and analytical interferences [4, 5] that may affect platelet aggregation. Learned societies suggest rejecting hemolytic specimens and noting other conditions in the result report [6]. An elevated bilirubin level also affects platelet function. Incubation of platelets with unconjugated bilirubin induces platelet morphological changes [7] and spontaneous platelet aggregation [8]. Literature has shown that the release of ADP by hemolyzed red blood cells can activate platelets [9–12]. Lipoproteins can interact with platelets [13, 14] and the non-aqueous lipids may affect light scattering, preventing any lightening of the tested PRP. Further studies have identified patients at risk of bleeding or increased platelet activation following the administration of lipid emulsion for malnutrition [15].

Additionally, the spectral properties of hemoglobin and bilirubin, with major absorbance peaks in the 400 to 600 nm range, may cause a spectrophotometric interference, as most of the aggregometers detect wavelengths from 430 to 740 nm. This study aims to empirically investigate the nature and extent of HIL interference on platelet aggregation using the newly introduced TA-8 V aggregometer equipped with a near infrared light source outside the absorbance range of hemoglobin, bilirubin, and lipids (wavelength ≥ 800 nm).

This study aims also to standardize the preanalytical phase of LTA by defining the concentrations of HIL beyond which a sample should be rejected. This is a particularly demanding requirement, especially for patients who travel from distant locations or rely on parenteral nutrition. This will be achieved through the analysis of PRP samples spiked with potential substances that mimic HIL interferences.

2 | Material and Methods

2.1 | Samples Preparation

The protocol has been validated by the ethical committee of our hospital (PADS21-32). Samples corresponded to remaining PRP collected from individuals referred to our center for a hemostasis exploration and prepared following the ISTH recommendations [6]. Individuals had no known platelet disorder and were not undergoing any drugs known to affect platelet function. Other inclusion criteria were: male or female, aged between 18 and 65 years old, and a platelet count between 150 and 450×10^9/L. Briefly, blood was collected after a short rest period with minimal or no venostasis using a needle of at least 21 gauge. It was drawn into polypropylene tubes with 109 mM sodium citrate after discarding the first 3–4 mL of blood. PRP was prepared by centrifuging blood samples at $200 \times g$ for 10 min at ambient temperature without using a break. PPP was prepared by centrifuging whole blood, or the tubes of blood from which PRP was removed, at ambient temperature at $2500 \times g$ for 15 min. A PRP platelet count $> 150 \times 10^9$/L was required. No PRP adjustment was done. PRP was rested at room temperature for 15 min. LTA studies were completed within a maximum of 4 h after blood sampling and 2.5 h after PRP preparation.

2.2 | Platelet Activators

Adenosine diphosphate (ADP) stock solution (Ref 86,923, Stago, Asnières, France) was reconstituted with 2 mL distilled water per vial (100 μmol/L), aliquoted, and stored at −20°C until use. For the experiments, ADP was diluted with distilled water to a concentration of 50 μmol/L and used at a final concentration of 5 μmol/L in the PRP (30 μL activator in 270 μL PRP).

Collagen stock solution (Ref 86,924, Stago, Asnières, France) was reconstituted with 0.5 mL distilled water per vial (1 mg/mL), aliquoted, and stored at −20°C until use. For the experiments, collagen was diluted with distilled water to a concentration of 20 μg/mL solution and used at a final concentration of 2 μg/mL in the PRP (30 μL activator in 270 μL PRP).

The concentrations used are intermediate concentrations commonly used to diagnose platelet dysfunction [6, 16].

2.3 | Red Blood Cell Hemolysate Preparation (RBCH)

Residual EDTA fresh whole blood from different individuals referred to our center as described above was centrifuged at $250 \times g$ for 15 min to remove plasma, platelets, and leukocytes. The red cell pellet was then washed at least 4 times with saline (NaCl 0.15 M) 1:1 volume by centrifugation at $2000 \times g$ for 10 min. The supernatant was discarded and the red blood cell pellet was lysed by adding a hypotonic solution (distilled water) 1:1 followed by vortexing. For improving hemolysis, the solution was then incubated at 60°C for 1 h and frozen overnight at −20°C. The hemolysate was thawed and centrifuged at $3000 \times g$ for 20 min at 4°C to remove cell debris. The top 1/3 volume of the tube constituted the red blood cell hemolysate stock solution, which was stored at 2°C–8°C for up to 1 week. Complete blood count and hemoglobin (Hb) concentration were measured using the XN-10 automated hematology analyzer (Sysmex). The final tested concentrations were 0, 0.3, 0.6, 2, 5, 10, and 20 g/L of Hb. When applied, 0.1 U/mL apyrase (Apyrase ADP-Premium ref. PY0627200, Agro-Bio, La Ferté Saint-Aubin, France) was incubated with the RBCH for 30 min at 37°C before addition to PRP.

2.4 | Bilirubin Solution Preparation

A stock solution of 8 g/L bilirubin was prepared by reconstituting unconjugated bilirubin (Ref B4126, Sigma-Aldrich/Merck, Saint Louis, MO) with 1 mL 0.1 N NaOH and kept in the dark. The stock solution was discarded after each experiment. The final concentrations tested were 0, 15, 60, 100, 180, 300, and 400 mg/L of bilirubin.

2.5 | Lipid Solution Preparation

The Intralipid 20% solution is a purified soybean oil-based lipid emulsion containing mainly long-chain triglycerides (200 mg triglycerides/mL) and also egg phospholipids and glycerol (Fresenius Kabi, Bad Homburg, Germany). The Intralipid

TABLE 1 | Maximal intensity obtained at the different concentrations of hemoglobin (A), bilirubin (B), and intralipids (C).

(A) Hemolysis	Hemoglobin concentration (g/L)	*n*	Mean ± SD
Spontaneous aggregation	0	22	3 ± 2
	0.3	22	4 ± 3
	0.6	22	3 ± 2
	2	22	9 ± 14
	5	22	18 ± 23
	10	5	43 ± 31
	20	5	41 ± 25
Spontaneous aggregation with apyrase	0	16	3 ± 2
	0.3	16	2 ± 2
	0.6	16	2 ± 1
	2	16	2 ± 1
	5	16	2 ± 1
	10	5	3 ± 2
	20	5	6 ± 5
ADP 5 μmol/L	0	11	81 ± 7
	0.3	11	81 ± 17
	0.6	11	77 ± 14
	2	11	74 ± 14
	5	11	72 ± 12
	10	5	63 ± 16
	20	5	64 ± 24
ADP 5 μmol/L with apyrase	0	11	84 ± 9
	0.3	11	81 ± 9
	0.6	11	78 ± 10
	2	11	78 ± 11
	5	11	78 ± 12
	10	5	83 ± 14
	20	5	68 ± 28
Collagen 2 μg/mL	0	10	85 ± 6
	0.3	10	81 ± 6
	0.6	10	79 ± 6
	2	10	77 ± 6
	5	10	79 ± 8
	10	5	71 ± 13
	20	5	65 ± 15
Collagen 2 μg/mL with apyrase	0	10	83 ± 5
	0.3	10	81 ± 7
	0.6	10	80 ± 7
	2	10	78 ± 8
	5	10	76 ± 20
	10	5	67 ± 27
	20	5	58 ± 27
(B) Icterus	**Bilirubin concentration (mg/L)**	***n***	**Mean ± SD**
Spontaneous aggregation	0	10	5 ± 2
	15	10	5 ± 2
	60	10	4 ± 2
	100	10	6 ± 3
	180	10	9 ± 4
	300	7	11 ± 6
	400	7	14 ± 9
ADP 5 μmol/L	0	11	81 ± 7
	15	11	77 ± 11
	60	11	80 ± 7
	100	11	81 ± 11
	180	11	82 ± 11
	300	5	78 ± 4
	400	5	78 ± 15
Collagen 2 μg/mL	0	11	78 ± 8
	15	11	80 ± 8
	60	11	81 ± 5
	100	11	87 ± 5
	180	11	85 ± 10
	300	5	76 ± 9
	400	5	76 ± 12
(C) Lipemia	**Intralipid concentration (g/L)**	***n***	**Mean ± SD**
Spontaneous aggregation	0	10	3 ± 2
	0.5	10	3 ± 2
	1	10	4 ± 2
	1.5	10	5 ± 4
	3	10	7 ± 7

TABLE 1 | (Continued)

(C) Lipemia	Intralipid concentration (g/L)	*n*	Mean ± SD
ADP 5 μmol/L	0	10	84 ± 5
	0.5	10	91 ± 9
	1	10	94 ± 13
	1.5	10	102 ± 7
	3	10	104 ± 10
Collagen 2 μg/mL	0	10	87 ± 5
	0.5	10	90 ± 8
	1	10	93 ± 14
	1.5	10	103 ± 4
	3	10	94 ± 17

Abbreviation: SD, standard deviation.

solution was directly mixed with the PRP to obtain the final tested concentrations of 0, 0.5, 1, 1.5, and 3 g/L (triglycerides equivalent). NaCl 0.15 M was added to the PRP to compensate for volume variations. Samples were tested immediately after homogenization.

The concentration range used is broader for hemoglobin and bilirubin than for lipids. For lipids, we encountered significant issues with homogenization beyond 4 g/L, which led us to limit the range of tested concentrations to 3 g/L. However, this concentration corresponds to levels observed in cases of familial hypertriglyceridemia.

2.6 | Light Transmission Aggregometry (LTA)

LTA was carried out using the TA-8 V aggregometer (Stago) in compliance with the aggregometer user manual. TA-8 V is an eight-channel aggregometer, equipped with a near infrared emitting diode of wavelength > 800 nm and receiving photodiode measuring the increase in light transmission during the platelet aggregation process. At this wavelength, the selected HIL concentrations are those that exhibit the lowest absorbance, as indicated in the aggregometer manufacturer's leaflet.

For each sample, the determination of 100% transmission was conducted using PPP, while the determination of 0% was conducted using unstimulated PRP. The recording time for LTA was set at 600 s. The number of samples used for each condition is specified in Table 1. At least 5 different PRP samples were tested for each condition. Maximum platelet aggregation intensity (MaxInt) and velocity (Vel) were recorded.

For each tested substance (RBCH, bilirubin, lipids), a control condition without the substance was tested in parallel. The same volume of RBCH, lipids, and bilirubin was added in all conditions, including the control condition, to account for the dilution effect and to ensure consistency across the conditions.

2.7 | Statistical Analysis

The data were analyzed using GraphPad Prism 7.0. Continuous variables were expressed as mean and standard deviation (SD). The coefficient of variation (CV) was calculated to assess MaxInt interindividual variability. Statistical differences of MaxInt and Vel across the HIL concentrations were determined using the non-parametric repeated measures Wilcoxon test. Statistical significance was assigned at a p-value of 0.05.

3 | Results

3.1 | Hemolysis Condition

RBHC was added to PPP and PRP at varying concentrations ranging from 0 to 20 g/L (Figure 1A). A spontaneous aggregation of platelets was observed from 2 g/L RBCH ($p < 0.01$) (Figure 1B). MaxInt between the condition without RBCH and the two highest concentrations reached 3% ± 2% vs. 43% ± 31% and 41% ± 25%, respectively (Table 1A). We hypothesized that ADP released during RBCH preparation might be causing spontaneous aggregation. Therefore, RBCH was incubated with 0.1 U/mL of apyrase for 30 min at 37°C. Apyrase acts by hydrolyzing ADP released from RBC into adenosine monophosphate. Apyrase-treated RBCH prevented the increase in MaxInt and Vel regardless of the RBCH concentration (Figure 1C). Values at 5 and 10 g/L RBCH in the presence of apyrase were equivalent to those without RBCH (3% ± 2% vs. 2% ± 1% and 3% ± 2%, respectively, Table 1A).

In order to avoid any interference with endogenous released ADP, we then examined concentration-response curves of apyrase-treated RBCH in PRP activated with 5 μmol/L ADP. Addition of apyrase reduced interindividual variability in comparison to the condition without apyrase (CV ranged from 17% to 21% for 0.3 to 5 g/L RBCH, and from 11% to 15% for 0.3 to 5 g/L apyrase-treated RBCH, $n = 11$). A subtle decrease in MaxInt was observed at 0.6 and 2 g/L RBCH ($p < 0.01$) (Figure 1D and Table 1A). This decrease was more pronounced for Vel (Figure 1D).

We replicated the same approach with LTA induced by 2 μg/mL collagen. The addition of apyrase-treated RBCH slightly reduced MaxInt at 2 g/L RBCH ($p < 0.01$) (Figure 1E). Vel was significantly reduced from 0.6 to 5 g/L apyrase-treated RBCH ($p < 0.01$) (Figure 1E and Table 1A).

3.2 | Icteric Condition

Bilirubin was added to PPP and PRP at increasing concentrations ranging from 0 to 400 mg/L (Figure 2A). We verified beforehand that the NaOH buffer not enriched with bilirubin did not affect platelet aggregation. Comparable MaxInt was obtained after the addition of equivalent volumes of 0.1 N NaOH and 0.15 M NaCl to PRP (spontaneous aggregation MaxInt at 12% ± 2% vs. 10% ± 5%; MaxInt in response to ADP at 92% ± 4% vs. 85% ± 9%; respectively; MaxInt in response to collagen at 82% ± 7% vs. 87% ± 4%, respectively). No spontaneous aggregation occurred for the first three concentrations, and a moderate increase in light transmission occurred at the three highest concentrations with a significant

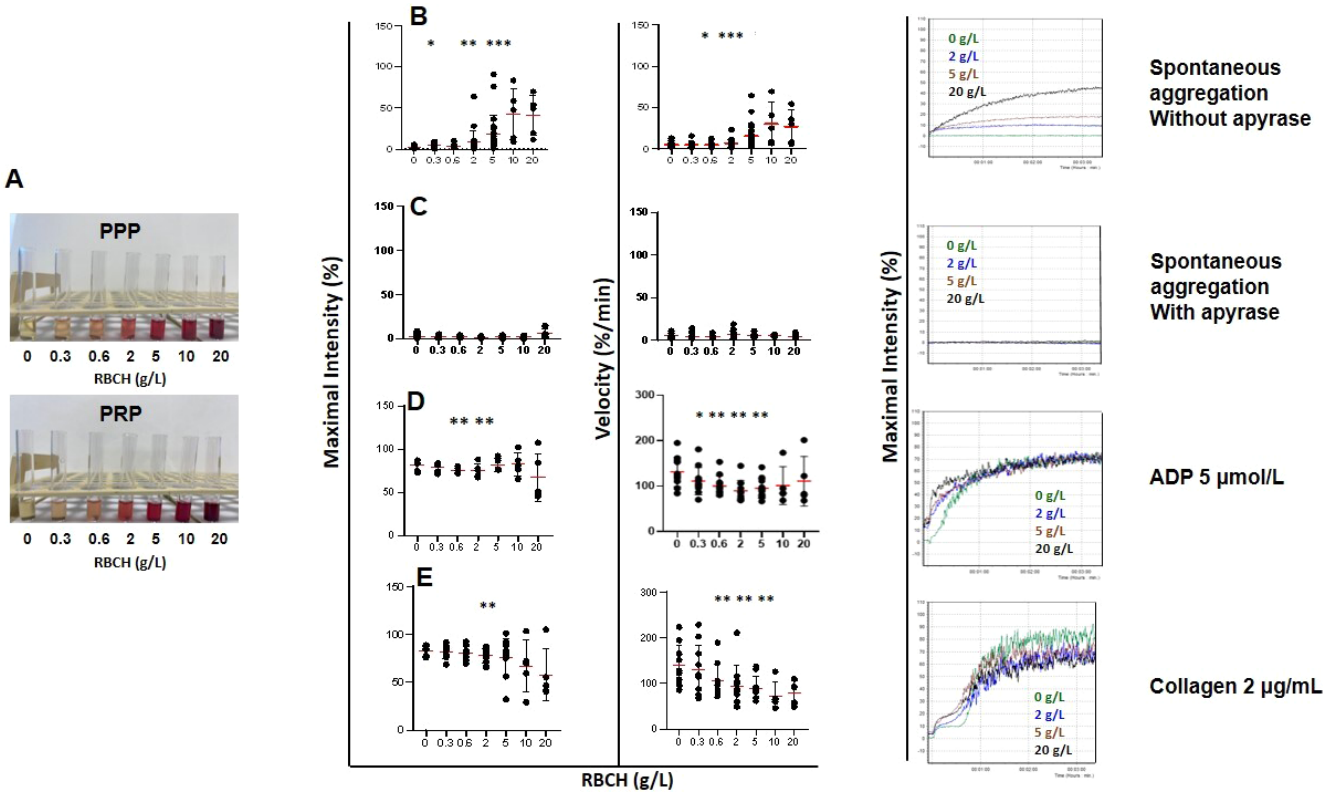

FIGURE 1 | Impact of red blood cell hemolysate on light transmission aggregometry. (A) RBCH was added at increasing concentrations (0 to 20 g/L) to platelet-poor and platelet-rich plasmas. (B) Maximal intensity (%) and velocity (%/min) were recorded during spontaneous platelet aggregation. The same samples were also tested in presence of apyrase (spontaneous aggregation) (C). Maximal intensity (%) and velocity (%/min) were recorded in response to 5 µmol/L ADP (D) and 2 µg/mL collagen (E). Representative platelet aggregation curves are shown for each condition. Maximal intensity and velocity obtained at each concentration of RBCH have been compared to values obtained without RBCH using the non-parametric repeated measures Wilcoxon test, *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$. PPP, platelet-poor plasma; PRP, platelet-rich plasma; RBCH, red blood cell hemolysate.

increase in MaxInt ($p<0.05$ for 300 mg/mL and $p<0.01$ for 180 and 400 mg/mL). A minor increase in Vel was observed at 100 mg/mL bilirubin and above (Figure 2B and Table 1B). MaxInt and Vel in response to ADP were not affected by increasing concentrations of bilirubin (Figure 2C): MaxInt between the condition without bilirubin and the two highest bilirubin concentrations was at 81% ± 7% vs. 78% ± 4% and 78% ± 15%, respectively (Table 1B). Subtle variation was observed in response to collagen (Figure 2D). A mild difference in MaxInt was only observed at 100 mg/L bilirubin ($p<0.01$) (Table 1B). Vel was not affected by the first five concentrations of bilirubin.

3.3 | Lipemic Condition

The 20% Intralipids solution was added to PPP and PRP at increasing concentrations ranging from 0 to 3 g/L (Figure 3A). In the absence of activator, a mild increase in light transmission occurred for the three highest concentrations (Figure 3B and Table 1C).

The presence of lipids significantly increases MaxInt in response to ADP 5 µmol/L starting at 0.5 g/L and intensifying at the two highest concentrations (MaxInt at 1.5 and 3 g/L vs. 0 g/L lipids: 102% ± 7%, 104% ± 10% vs. 84% ± 5%, $n=10$, $p<0.01$ and $p<0.01$ respectively) (Figure 3C and Table 1C). When analyzing individual values, we observed a progressive increase in MaxInt values above 100% with increasing lipid concentration. While only two values were above 100% at 0.5 g/L, this number reached 8 out of 10 at 3 g/L. There was a trend towards higher MaxInt values in response to collagen with higher lipid concentrations, with a significant difference observed for 1.5 g/L only ($p<0.01$) (Figure 3D and Table 1C). Similar to ADP, we observed a progressive increase in MaxInt above 100% with increasing lipid concentration (8 out of the 10 tested samples were above 100% at 1.5 g/L). Vel in response to ADP or collagen was not significantly affected by lipids (Figure 3C).

However, we verified that the absolute absorbance of PPP and PRP is not affected by the addition of RBCH, bilirubin, and intralipids. The range of the absolute difference in absorbance between PRP and PPP varies between 2727 ± 113 AU and 2791 ± 122 AU for RBCH; between 2749 ± 72 AU and 2847 ± 36 AU for bilirubin; and between 2769 ± 43 AU and 2846 ± 51 AU for lipids.

4 | Discussion

HIL are common conditions in clinical laboratories and have been reported to affect up to 19.5%, 0.3%, and 0.3%, respectively, of samples referred to hemostasis laboratories [17].

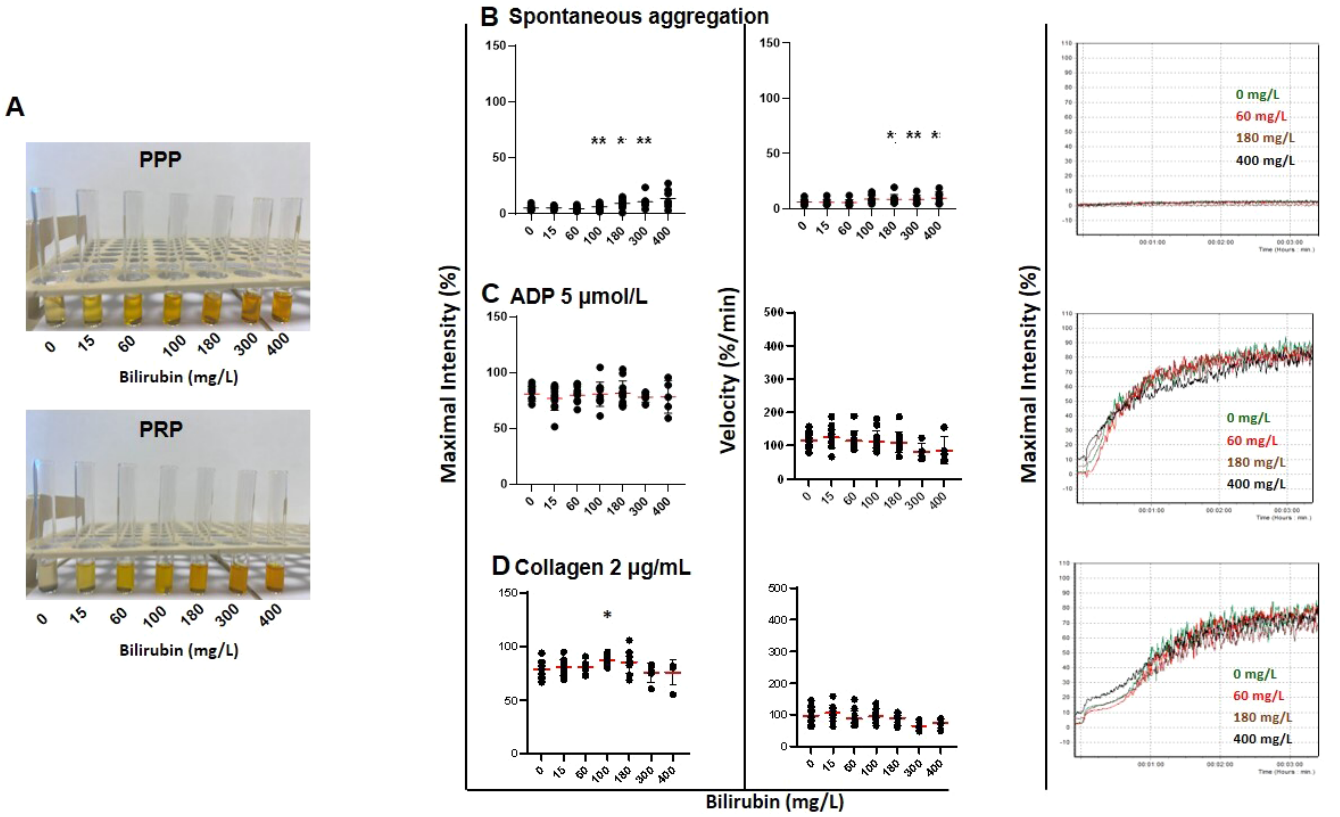

FIGURE 2 | Impact of unconjugated bilirubin on light transmission aggregometry. (A) Bilirubin was added at increasing concentrations (0 to 400 mg/L) to platelet-poor and platelet-rich plasmas. Maximal intensity (%) and velocity (%/min) were recorded during spontaneous platelet aggregation (B), in response to 5 µmol/L ADP (C) and 2 µg/mL collagen (D). Representative platelet aggregation curves are shown for each condition. Maximal intensity and velocity obtained at each concentration of bilirubin have been compared to values obtained without bilirubin using non-parametric repeated measures Wilcoxon test, $*p < 0.05$, $**p < 0.01$. PPP, platelet-poor plasma; PRP, platelet-rich plasma.

Hemolysis is the most prevalent preanalytical error in laboratory medicine. It can occur both in vivo and in vitro. Laboratories must take careful steps to confidently rule out in vivo hemolysis. In contrast, in vitro hemolysis is highly preventable. A multitude of potential mechanisms can lead to in vitro hemolysis, including overly vigorous blood draw techniques, collection from non-standard sites, and failure to adhere to proper conditions during transport and sample preparation [18]. Additionally, factors such as thrombin production during a challenging blood draw can exacerbate the effects of hemolysis on platelet function.

LTA remains widely used but can be limited in cases of a high degree of colored or particulate interferences in plasma samples that can lead to erroneous results. Here, we aim to evaluate the in vitro consequences of HIL on platelet aggregation in healthy samples using a novel aggregometer, TA-8 V, based on infrared detection technology aimed at reducing optical interferences. In this study, each sample was tested under both non-HIL conditions and conditions mimicking HIL interferences.

For over 50 years, the ability of hemolysis to activate platelets both in vivo and in vitro has been documented [9, 10, 12]. Our results suggested that samples with hemolysis levels exceeding 0.6 g/L RBCH appear risky to be used in clinical practice, as it may result in spontaneous aggregation and heightened variability in response to 5 µmol/L ADP amongst individuals. Nevertheless, very high concentrations of hemoglobin (above 1 g/L) are rarely observed in clinical practice. Spontaneous aggregation was found to be due to the presence of ADP released by the hemolysate, as apyrase, which metabolizes ADP to adenosine monophosphate, has fully prevented spontaneous aggregation. This phenomenon has already been observed with the Multiplate aggregometer based on impedance and in whole blood [10], indicating that it occurs independently of the detection system used. In the presence of apyrase, MaxInt remained very low whatever the RBCH concentration indicating that the TA-8 V instrument is, as expected, insensitive to the potential interference from the red-colored hemoglobin substance. After blocking this biological interference with apyrase, we evaluated the platelet response to activators. We showed that the RBCH has a more pronounced interference on Vel than on MaxInt. Our results suggest that, under in vitro conditions, it is possible to measure MaxInt in the presence of RBCH, provided that endogenous ADP is neutralized. The decrease in Vel starting at 0.3 g/L is also observed in the absence of apyrase; its explanation needs further investigation and may be related to transitory P2Y1 desensitization [19]. This effect is unlikely to be due to spectral interference of hemolysate, as plasma infrared spectral profile is not affected by hemolysate until 2 g/L [20]. It is suggested that neutralization of the ADP is likely to reveal an inhibitory effect of the hemolysate on platelet aggregation rate. Overall, in clinical

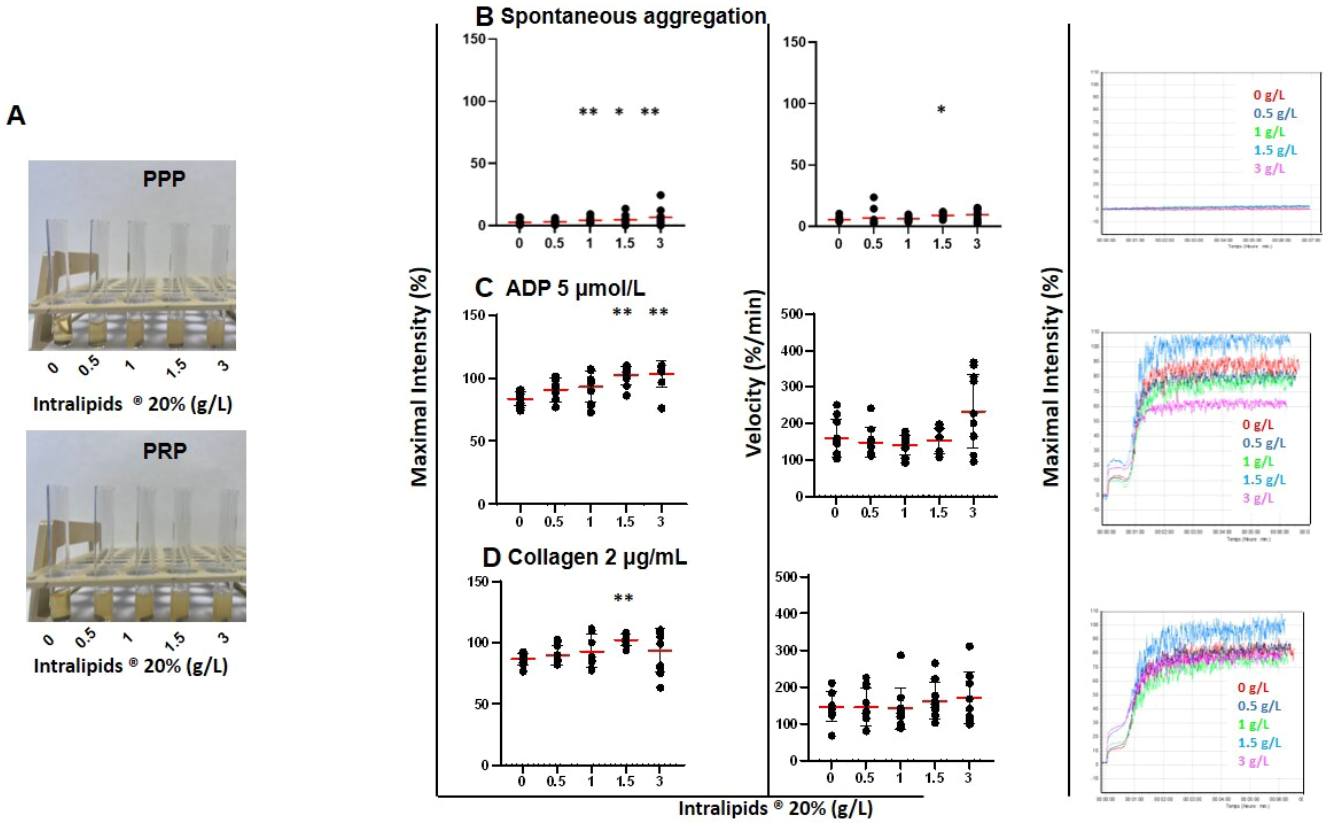

FIGURE 3 | Impact of Intralipid on light transmission aggregometry. (A) Intralipid was added at increasing concentrations of Intralipids (equivalent triglycerides 0 to 3 g/L) to platelet-poor and platelet-rich plasmas. Maximal intensity (%) and velocity (%/min) obtained at each concentration were recorded during spontaneous platelet aggregation (B), in response to 5 µmol/L ADP (C) and 2 µg/mL collagen (D). Representative platelet aggregation curves are shown for each condition. Maximal intensity and velocity obtained at each concentration of Intralipid have been compared to values obtained without Intralipid using non-parametric repeated measures Wilcoxon test, $*p < 0.05$, $**p < 0.01$. PPP, platelet-poor plasma; PRP, platelet-rich plasma.

practice, rejection of grossly hemolysed samples may appear reasonable. However, in the case of severe platelet dysfunction expected to be associated with absence of response to any activators, as in Glanzmann thrombasthenia, the release of ADP during hemolysis may not be able to induce platelet aggregation. This means that there are risks arising from sample rejection which must be balanced against the risks of not performing a diagnosis on hemolysed samples. Unfortunately, our study did not include the use of pathological samples.

Unconjugated bilirubin is a yellow-colored compound produced in the body as a result of heme catabolism [21]. Levels of unconjugated bilirubin greater than 50 µmol/L (30 mg/mL) are associated with icterus. Previous research has shown that unconjugated bilirubin causes spontaneous aggregation of human washed platelets in a concentration-dependent manner at physiological levels starting from 2 mg/L (3.4 µmol/L) and peaking at 20 mg/L (34 µmol/L). This aggregation can be inhibited in the presence of albumin, the scavenger of unconjugated bilirubin in plasma, suggesting that free unconjugated bilirubin concentration in plasma is likely too low under normal conditions to activate platelets but may become relevant in cases of bilirubin overload [8]. We have further confirmed this hypothesis by observing a minor increase in MaxInt that likely may correspond to a mild spontaneous platelet aggregation at concentrations exceeding 100 mg/L (171 µmol/L).

Our study found that unconjugated bilirubin does not affect ADP or collagen-stimulated platelet aggregation measured with an infrared source light until concentrations reach 100 mg/mL (171 µmol/L). Our findings align closely with those of Kundur et al. [22] who demonstrated a minimal impact of unconjugated bilirubin on platelet aggregation using PRP up to 200 µmol/L (117 mg/mL) with a time-dependent effect [22]. Our results show that the performance of TA-8 V is not influenced by the presence of jaundice up to high concentrations of free bilirubin (400 mg/L).

"Opalescent" plasma indicates the presence of triglycerides above 3 to 4 g/L. In the case of severe metabolic abnormalities, triglyceride levels are even higher, leading to a milky appearance referred to as lactescent serum with triglyceride levels up to 25/30 g/L. Intralipid is an emulsion of soybean triacylglycerol and egg phospholipids which is similar to chylomicrons with regard to particle size and metabolic fate [23, 24] and was loaded into samples to mimic hypertriglyceridemia. Intralipid did not influence Vel; however, it significantly increased MaxInt (e.g., 84% + 5% for the condition without Intralipid vs. 102% ± 7% for the condition 1.5 g/L Intralipid in response to ADP). At this concentration, most of the MaxInt values were above 100%. The observed increase in MaxInt could be attributed to the interaction of lipid particles with the platelet aggregates, as described for VLDL and chylomicrons [13], resulting in a reduction in

sample opacity compared to Intralipid-enriched PPP. This needs, however, to be confirmed. Moreover, hyperlipidemic plasma has been shown to translate into specific absorbance wavelengths in the mid-infrared range, interfering with the infrared spectral profile [20]. This may cause erroneous 0% with increasing lipid concentration. In addition, Intralipid dilution may alter the emulsifier/water/oil ratio leading to a heterogeneous solution and a change in light transmission, with possibly higher light transmission in PRP than in PPP. Our results differed from those obtained after intravenous infusion of Intralipid into humans, where LTA induced by collagen or ADP was significantly reduced by up to 40% and 45%, respectively, 3 h post- infusion [25]. Both components of Intralipid (liposomes and triglyceride-phospholipid particles) were able to decrease in vitro platelet aggregation of diluted PRP incubated with the different emulsions for 2 h. Similarly, the incubation of washed rat platelets with Intralipid for 30 min reduced platelet aggregation in response to 2.5 μmol/L ADP, 25 μg/mL collagen, and 0.05 U/mL thrombin, at concentrations as low as 1 g/L [26]. These results are attributed to the general belief that emulsion particles may facilitate the removal of unesterified cholesterol from the platelet plasma membrane by acting as an acceptor of unesterified cholesterol [27, 28]. In our experiments, platelet aggregation was assessed immediately after lipid loading, likely preventing cholesterol extraction from the membranes and attenuation of aggregation.

Overall, our results indicate that lipid emulsion moderately increases platelet aggregation, indicating that the performances of the TA-8 V instrument are influenced by hypertriglyceridemia starting at a moderate concentration (1.5 g/L).

Our study has several limitations. It was based on the enrichment of PRP with various products that only partially replicate pathogenic conditions observed in vivo. Additionally, the absence of incubation time used prevented us from evaluating the effects caused by chronic platelet impregnation in a pathological environment in vivo. A lack of statistical power may explain the absence of differences in MaxInt and Vel for the two highest concentrations in some conditions (e.g., spontaneous aggregation with RBCH). In addition, our study did not include patients with platelet dysfunction.

5 | Conclusion

Reporting accurate results is a critical concern for clinical laboratories. It is therefore important that the laboratory staff be aware of potentially instrument-relevant bias caused by interferences. HIL is a group of the most commonly observed sources of interferences. Our findings suggest that platelet aggregation testing using the TA-8 V instrument is not significantly impacted by icterus within the specified ranges in healthy individuals. However, grossly hemolyzed samples (exceeding 0.6 g/L RBCH) have to be rejected to avoid biological interference with ADP released from red blood cells. Our results for hyperlipidemic samples should be interpreted with caution due to potential issues related to the mixing of PRP and intralipid.

Further research is needed to confirm these results in patients with platelet dysfunction.

Author Contributions

This work was supported by the French national reference center for genetic platelet disorders. M.-C.A. conceived and designed the study. M.I.-K. and G.Z. performed the experiments. M.-C.A., M.I.-K., and G.Z. conducted thorough analyses and interpretation of the data. P.O. and K.C. provided the TA-V8 and contributed to the generation of LTA curves. All co-authors revised and approved the manuscript.

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Conflicts of Interest

P.O. and K.C. are employees in Diagnostica Stago. The other authors declare no conflicts of interest.

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