Investigations of Platelet Function in Whole Blood with BAPA as Anticoagulant

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Keywords

BAPA · Storage time · Platelet function · Whole blood

Summary

Background: Citrate anticoagulation of blood results in non-physiologically low calcium concentration and rapid deterioration of platelet viability. Benzylsulfonyl-D-Arg-Pro-4-amidinobenzylamide (BAPA) as anticoagulant maintains the physiological calcium level and seems to retain platelet function (PF) over a time period of at least 24 h. We evaluated PF in BAPA-anticoagulated peripheral whole blood (WB) between 0.5 and 30 h after blood collection. **Methods:** In WB from 21 healthy volunteers (15 women, 6 men, age range 19–57 years) platelet aggregation (PA) was determined by impedance method and ATP release by luminometry. Platelet response was tested by ADP (10 and 20 $\mu\text{mol/l}$) and collagen (1 and 2 $\mu\text{g/ml}$) between 0.5 and 30 h after blood collection. **Results:** Parameters of ADP-induced PA showed stable values until 6.5 h after blood collection followed by a significant decline. PA in response to collagen was stable up to 25 h of storage. ATP release induced by collagen displayed a continuous, significant decrease over time. **Conclusion:** Preservation of platelet response in BAPA-anticoagulated blood depends on the applied agonist showing that collagen-induced PA is more stable compared to ADP known as a weak agonist in WB.

Schlüsselwörter

BAPA · Lagerungszeit · Thrombozytenfunktion · Vollblut

Zusammenfassung:

Hintergrund: Die Antikoagulation von Vollblut (VB) mit Zitrat führt zu einer erniedrigten Kalziumkonzentration, wodurch die Thrombozytenüberlebenszeit eingeschränkt wird. Benzylsulfonyl-D-Arg-Pro-4-amidinobenzylamid (BAPA) als Antikoagulant hält die physiologische Kalziumkonzentration aufrecht und scheint eine ausreichende Plättchenfunktion über mindestens 24 h zu gewährleisten. Wir führten Plättchenfunktionsuntersuchungen in BAPA-antikoaguliertem VB durch, welches im Zeitraum zwischen 0,5 bis 30 h nach Blutabnahme gelagert wurde. **Methoden:** In VB-Proben von 21 gesunden Freiwilligen (15 Frauen, 6 Männer, Alter 19–57 Jahre) wurden die Plättchenaggregation (PA) mit Hilfe der Impedanzmethode und die ATP-Freisetzung mittels Luminometrie untersucht. Die PA wurde mit ADP (10 und 20 $\mu\text{mol/l}$) und Kollagen (1 und 2 $\mu\text{g/ml}$) nach einer Lagerungszeit von 0,5–30 h nach Blutentnahme induziert. **Ergebnisse:** Die Parameter der ADP-induzierten PA zeigten stabile Werte bis zu 6,5 h nach Blutentnahme gefolgt von einem signifikanten Abfall. Die Aggregation in Response auf Kollagen war bis zu einer Lagerung von 25 h stabil. Die ATP-Freisetzung induziert durch Kollagen zeigte einen kontinuierlichen, signifikanten Abfall. **Schlussfolgerung:** Die Erhaltung der Plättchenfunktion in BAPA-antikoaguliertem Blut ist abhängig vom verwendeten Agonisten, wobei sich die Aggregation mit Kollagen als stabiler im Vergleich zum schwächer wirksamen Agonisten ADP erweist.

Introduction

Testing of platelet function is essential for the detection of hereditary and acquired platelet function defects. As recently published, light transmission aggregometry (LTA) is the most commonly used method for this purpose in coagulation laboratories of German-speaking countries [1]. However, LTA is a time-consuming and technically challenging method affected by many pre-analytical and analytical variables. For this reason LTA should be performed only in highly specialized laboratories by expert personnel [2].

Sodium citrate is still the most commonly used blood anticoagulant for LTA in platelet-rich plasma (PRP) and for impedance aggregometry in whole blood (WB) [2, 3]. It acts by chelating extracellular Ca^{2+} , leading to a decrease in Ca^{2+} levels in the sample from about 1.2 to 0.1 mmol/l [4–6]. Due to the fact that platelets are dependent on Ca^{2+} for normal function, the platelet viability is markedly reduced in citrated blood which is a critical point with respect to the transportation time of samples to the coagulation laboratory [7]. In many laboratories it is the rule that the investigation of aggregometry in PRP and in WB should start 15–30 min after blood collection and must be completed within 3–4 h [2, 3, 8, 9]. Within this tight time frame the limited platelet viability in citrate-anticoagulated blood does not allow the shipment of samples from patients with suspected platelet function defects to distant coagulation laboratories for sophisticated analysis.

Anticoagulants that maintain the physiological concentration of divalent cations (Ca^{2+} , Mg^{2+}) in the samples, such as the direct thrombin inhibitor hirudin, seem to be more suitable as anticoagulant for investigations of platelet aggregation [10, 11]. However, hirudin has also some drawbacks, for instance the weak agonist-induced second wave of the aggregation curve is absent which is an important parameter for detection of platelet function disorders [2]. In addition, analysis of platelet release reaction displays some limitations in hirudin-anticoagulated blood [12–14].

Recently, the synthesis of a new anticoagulant, benzylsulfon-D-Arg-Pro-4-amidinobenzylamide (BAPA), was described which acts as dual inhibitor of thrombin and factor Xa and does not affect the physiological Ca^{2+} level [15, 16]. Blood anticoagulation with BAPA is reported to retain platelet function over a time period of at least 24 h [5, 6]. Platelet aggregation testing in WB represents an established diagnostic method in some clinical laboratories. In the pediatric setting it has the advantage of the relatively low amount of blood needed. This prompted us to evaluate platelet function in BAPA-anticoagulated WB samples over a time period of 30 h after blood collection.

Material and Methods

Blood Collection

Blood was aspirated by peripheral venous puncture in 21 apparently healthy volunteers (15 females and 6 males; age range 19–57 years; mean age 32 years) who gave their informed consent in accordance with the Ethical Hospital Review Board approval. They had not taken any medication influencing platelet function for at least 14 days before testing. After discharge of the first 3 ml of blood specimens were anticoagulated with BAPA (final concentration 50 $\mu\text{mol/l}$; Thrombovette® Xipla, Probe and Go, Osburg, Germany) and stored until measurement at room temperature ($22 \pm 2^\circ\text{C}$). Platelet response was measured 0.5, 6.5, 25 or 30 h after blood collection.

Platelet Response Measurement

Platelet aggregation was determined by impedance method and ATP release by luminometry using a two-channel Chrono-Log lumi-aggregometer (type 560-CA, Chrono-Log Corporation, Havertown, PA, USA) in accordance with the manufacturer's instructions and the description in the literature [17]. Aggregation and ATP release were induced by ADP at final concentrations of 10 and 20 $\mu\text{mol/l}$ and by collagen (type I collagen from equine tendons) at 1 and 2 $\mu\text{g/ml}$. After addition of agonist, aggregation curves were recorded for 6 min. Within this time the maximal aggregation was determined and expressed in ohms (Ω). ATP release curves were recorded until the peak was reached, and the maximal release was calculated in nmol by adding an ATP standard to a separate sample. Both agonists and the ATP standard were purchased from Chrono-Log Corporation. Parameters of aggregation and ATP release curves were determined by AGGRO/LINK software (Version 4.0; Chrono-Log Corporation).

Statistics

Data were analyzed using Kolmogorov-Smirnov test to calculate the values for normality. Since all values were normally distributed, paired data were compared using T-test with Holm adjustment of the p values. Any two-tailed probability of the corrected p-value below 0.05 was considered significant. Statistical analyses were performed using SPSS for Windows software, version 17.0.0 (SPSS Inc., Chicago, IL, USA), and diagrams were constructed using GraphPad Software, version 5.0 (GRAPHPAD Software Inc., San Diego, CA, USA).

Results

ADP-Induced Platelet Aggregation

Platelet aggregation by ADP at 10 and 20 $\mu\text{mol/l}$ showed a slight, non-significant decline within the time interval from 0.5 to 6.5 h (ADP 10 $\mu\text{mol/l}$ from $14.0 \pm 3.1 \Omega$ to $12.1 \pm 3.7 \Omega$; ADP 20 $\mu\text{mol/l}$ $14.5 \pm 2.5 \Omega$ to $13.6 \pm 3.7 \Omega$) after blood collection (fig. 1 and 2). For both ADP concentrations a significant decrease of platelet aggregation after 6.5 h of storage time was shown.

Collagen-Induced Platelet Aggregation

Statistical analysis revealed that the collagen-induced platelet aggregation at 1 and 2 $\mu\text{g/ml}$ remained stable until 25 h. Thereafter, it displayed a significant decline (fig. 3 and 4). For collagen at 1 $\mu\text{g/ml}$ (2 $\mu\text{g/ml}$) mean values of $17.9 \pm 2.8 \Omega$ ($18.3 \pm 3.0 \Omega$) at 0.5 h, $16.8 \pm 2.1 \Omega$ ($17.4 \pm 2.6 \Omega$) at 6.5 h and $15.8 \pm 4.2 \Omega$ ($17.6 \pm 2.9 \Omega$) at 25 h were calculated.

Fig. 1. Platelet aggregation induced by 10 µmol/l ADP depending on the storage time. Data points represent the mean ($\pm$ 95% CI) of 21 individuals. * p < 0.05; ** p < 0.01; *** p < 0.001; n.s. = not significant.

Fig. 4. Platelet aggregation induced by 2 µg/ml collagen depending on the storage time. Data points represent the mean ($\pm$ 95% CI) of 21 individuals. * p < 0.05; ** p < 0.01; *** p < 0.001; n.s. = not significant.

Fig. 2. Platelet aggregation in response to ADP at 20 µmol/l depending on the storage time. Data points represent the mean ($\pm$ 95% CI) of 21 individuals. * p < 0.05; ** p < 0.01; *** p < 0.001; n.s. = not significant.

Fig. 5. ATP-release induced by 1 µg/ml collagen depending on the storage time. Data points represent the mean ($\pm$ 95% CI) of 21 individuals. * p < 0.05; ** p < 0.01; *** p < 0.001; n.s. = not significant.

Fig. 3. Platelet aggregation induced by 1 µg/ml collagen depending on the storage time. Data points represent the mean ($\pm$ 95% CI) of 21 individuals. * p < 0.05; ** p < 0.01; *** p < 0.001; n.s. = not significant.

Fig. 6. ATP-release induced by 2 µg/ml collagen depending on the storage time. Data points represent the mean ($\pm$ 95% CI) of 21 individuals. * p < 0.05; ** p < 0.01; *** p < 0.001; n.s. = not significant.

Collagen-Induced ATP Release

For the ATP release at both collagen concentrations a significant decrease during the first 6.5 h (collagen 1 $\mu\text{g/ml}$ 0.47 ± 0.24 nmol at 0.5 h to 0.3 ± 0.18 nmol at 6.5 h; collagen 2 $\mu\text{g/ml}$ 0.87 ± 0.19 nmol at 0.5 h to 0.74 ± 0.26 nmol at 6.5 h) was detected which was followed by a slow further decline until 30 h of blood storage (fig. 5 and 6). For collagen at 1 $\mu\text{g/ml}$, ATP release remained stable from 6.5 to 30 h, and for collagen at 2 $\mu\text{g/ml}$ ATP release decreased significantly from 6.5 to 30 h.

Discussion

Up to now, only a limited number of studies have been published regarding the determination of platelet function in BAPA-anticoagulated blood samples [5, 6, 18, 19]. Here we demonstrate that the preservation of platelet response in BAPA-anticoagulated WB seems to depend on the agonist applied. Platelet aggregation determined by the impedance method induced by collagen which is known as a strong agonist in whole blood was more stable over time compared to ADP being a weak agonist.

Our observations of declining ADP-induced platelet aggregation in BAPA-anticoagulated WB over time are supported by data reported from Kaiser et al. [18] and Hellis et al. [19]. Kaiser et al. [18] revealed a loss of aggregation response to ADP at 5 $\mu\text{mol/l}$ in BAPA-anticoagulated WB between 8 and 12 h. Hellis et al. [19] analyzed ADP-induced platelet aggregation at 6.4 $\mu\text{mol/l}$ in BAPA-anticoagulated WB and found a significant decrease of platelet response at 12 h after blood collection compared to the aggregation at 2 h. For ADP at 5 $\mu\text{mol/l}$ in BAPA-anticoagulated PRP they could show a significant decrease within 22 h after blood collection [19]. These findings are in line with our observation of a decreased platelet response by the weak agonist ADP in a nearly time-dependent manner.

These findings differ from two studies which did not detect a significant decrease of ADP-induced aggregation at 10 $\mu\text{mol/l}$ after up to 32 h of storage [6] and at 6.5 $\mu\text{mol/l}$ after up to 24 h [5] following blood collection in BAPA-anticoagulated WB. However, our results contrast these findings as the final concentrations of 10 and 20 $\mu\text{mol/l}$ of ADP led to stable aggregation curves for only 6.5 h after blood collection. As in these two studies also BAPA-anticoagulated WB the impedance method for determination of aggregation and comparable ADP final concentrations were used, but the discrepancies to our results cannot be explained sufficiently. However, it should be mentioned that several methodological variables which are not given in detail in the papers, e.g. the storage conditions of the BAPA-anticoagulated blood, the speed of stirrer in the sample and others, may substantially influence results of aggregation testing.

Concerning collagen-induced aggregation and ATP release, Haubelt et al. [6] demonstrated that both parameters remained stable over a time period of up to 48 h after blood collection using a final concentration of 5 $\mu\text{g/ml}$ of collagen in BAPA-anticoagulated WB. The authors suggested that anticoagulation with BAPA enables the transport of blood specimens from distant locations for platelet function testing. The same conclusion was drawn by Hellstern et al. [5] who examined platelet function in BAPA-anticoagulated PRP and WB using collagen at final concentrations of 2 and 3.2 $\mu\text{g/ml}$ and demonstrated stable results between 2 and 24 h after blood collection [5]. Using collagen at 1 and 2 $\mu\text{g/ml}$ we also found stable aggregation curves up to 25 h of storage time. Thus, the stability of collagen-induced platelet aggregation and ATP release over time in BAPA-anticoagulated samples seems to be influenced by the applied final collagen concentration. The high final collagen concentration of 5 $\mu\text{g/ml}$ seems to retain platelet function in whole blood over a long time period (48 h), but it may be questionable if this concentration may allow the detection of platelet function defects.

The determination of ATP release from platelets displays an important diagnostic tool for the detection of platelet secretion disorders [3]. However, BAPA as inhibitor of thrombin does not allow the determination of thrombin-induced maximal ATP release. Taking into consideration, that we found a significant decrease of collagen-induced ATP release already within the first 6.5 h after blood collection, the determination of ATP release in BAPA-anticoagulated samples seems not to be of use.

Only studies using BAPA-anticoagulated samples obtained from healthy adults have been published so far. Therefore, the application of BAPA in routine coagulation laboratories needs further investigations, and the suitability of BAPA for determination of specific platelet function defects in patients with hereditary or acquired platelet disorders still has to be proven. From our point of view the shipment of samples to distant laboratories is a critical point due to the delay in platelet function testing which has well-known limitations. Therefore, we recommend the shipment of BAPA-anticoagulated blood only for testing of collagen-induced aggregation and in combination with a control sample.

Acknowledgement

The authors would like to thank E. Kuhlisch (Institute for Medical Informatics and Biometry at the University of Dresden, Germany) for his recommendations to the statistical data analysis.

Disclosure

The authors declared no conflict of interest.

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