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## Propyl gallate-induced platelet aggregation in patients with end stage renal disease – The influence of the hemodialysis procedure

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### Abstract

Platelet dysfunction is a well-established disturbance in hemodialysis (HD) patients. Propyl gallate is a synthetic platelet activator with the property to stimulate platelet aggregation. The aim of this study was to evaluate the influence of a single hemodialysis session on propyl gallate-induced platelet aggregation.

Thirty-nine HD patients were enrolled in the study and 20 healthy volunteers were used as controls. Cellulose diacetate (CD) dialysers were used in 20 patients and polysulfone (PS) dialysers in 19. HD was performed via an A-V fistula in 27 patients and via an intravenous catheter in 12. Erythropoietin was administered in 37 patients (epoietin-alpha in 24 and darbepoietin in 13). Thirty-four were receiving the low molecular weight heparin tinzaparin. Propyl gallate slide aggregometry was used for evaluating platelet aggregation.

In HD patients, platelet aggregation was impaired before as well as after the HD session. No effect of the HD procedure, type of vascular access, adequacy of HD or type of erythropoietin on the propyl gallate-induced platelet aggregation was detected. Platelet aggregation was higher when CD dialyser was used. A negative correlation between the time needed for platelet aggregation to occur and tinzaparin dose was found.

Propyl-gallate induced platelet aggregation in HD patients is impaired. Platelet aggregation was higher in patients dialyzed with CD membrane than in those dialyzed with PS membrane. The higher was the dose of tinzaparin, the higher the platelet aggregation. The clinical significance of the above results needs further evaluation.

**Keywords:** hemodialysis, platelet aggregation, propyl-gallate, erythropoietin, low molecular weight heparin

### Introduction

Chronic hemodialysis (HD) treatment improves uremia induced bleeding diathesis but only partially (1,2). The bleeding diathesis observed in HD patients is of multifactorial origin but platelet dysfunction induced by uremia seems to play a central role. Uremic thrombocytopeny is attributed to acquired defects in specific receptors that impair platelet binding to fibrinogen and von Willebrand factor (3,4), lower than normal content of ADP and serotonin in platelet  $\alpha$ -granules (5) and impaired arachidonate metabolism (6). Guanidinosuccinic acid, a low molecular weight uremic toxin related with an increase in NO

production by uremic vessels, is implicated in platelet dysfunction (7).

Recombinant human erythropoietin (rHuEPO) administration in HD patients improves bleeding diathesis because it increases red cells blood count and ameliorates blood rheology abnormalities (8,9). On the other hand, there is controversy regarding the effect of rHuEPO administration on platelet aggregation (9-16).

Heparin, the commonly used anticoagulant in HD procedure, activates platelets and increases platelet aggregation (18,19). A few studies have evaluated the effect of the more recently used low molecular weight heparins (LMWH) on platelet aggregation in HD patients (20,21). In non-uremic subjects LMWH seems to increase platelet aggregation, although to a lesser extend than unfractionated heparin (22-25).

Some authors have found out that HD procedure per se enhances (3,26) platelet activation and aggregation, while others reached different conclusions (27,28). Finally, the type of the dialysis membrane used seems to play an important role in platelet activation or aggregation during the dialysis session. However, the results of different studies are controversial (27, 29-31).

The aim of our study was to evaluate the impact of HD procedure and its different aspects on platelet aggregation. The impact of the medications commonly used in HD patients, i.e. rHuEPO and LMWH, on platelet aggregation, was also assessed. For this purpose the simple and inexpensive propyl gallate (PG) slide aggregometry test was used. This test has been shown to be more sensitive in detecting most of the abnormal platelet aggregation conditions than the commonly used platelet aggregating reagents (32). In addition, this procedure shows strong correlation with the bleeding time test (33). To our knowledge this is the first time that PG was used as platelet aggregation agonist in order to evaluate platelet aggregation in HD patients. Impairment in arachidonate metabolism, adenosine phosphate receptors or ADP and glycoprotein IIb/IIIa (GpIIb/IIIa) receptor availability can contribute to platelet dysfunction in HD patients (3-6). Propyl gallate can concurrently detect disorders in all these factors (34). This fact makes it probably the most suitable reagent for an overall evaluation of platelet aggregation in HD patients, in contrast with other commonly used platelet aggregation agonists like arachidonate, ADP, epinephrine, fibrinogen and others, which evaluate only specific platelet aggregation pathways (35).

## Patients and methods

### Patients

Thirty-nine patients (19 males, 20 females, mean age  $62 \pm 11$  years) with end stage renal disease undergoing chronic HD (mean HD duration  $63 \pm 61$  months) were studied. The cause of end-stage renal disease was primary glomerulonephritis in 13 patients, diabetes mellitus in 5 patients, hypertension in 4 patients, polycystic kidney disease in 3 patients, Alport's syndrome in 2 patients, analgesic nephropathy in 1 patient, primary amyloidosis in 1 patient, and unknown in 10 patients. Patients underwent regular HD with a bicarbonate buffer for 4 hours each day, 3 days a week. Vascular access was A-V fistula in 27 patients and a permanent double central venous catheter in 12 patients. Thirty-seven patients were receiving i.v. erythropoietin (rHuEPO), 24 epoietin-alpha (Eprex<sup>®</sup>; Johnson & Johnson, Manati, PR) and 13 darbepoietin-alpha (Aranesp<sup>®</sup>; Amgen, Thousand Oaks, CA). During treatment the LMWH tinzaparin sodium (Innohep<sup>®</sup>; Leo Pharmaceuticals, Ballerup, Denmark) was used for anticoagulation in 34 patients, unfractionated heparin (Heparin<sup>®</sup>, Leo Pharmaceuticals, Ballerup, Denmark) in 2 patients, while 3 patients did not receive anticoagulation. Cellulose diacetate (CD) dialysers (FB-T series<sup>®</sup>; Nipro Corporation, Osaka, Japan) were used in 20 patients and polysulfone (PS) dialysers (F low-flux series<sup>®</sup>; Fresenius Medical Care, Bad Homburg, Germany) in the other 19 patients.

Twenty healthy volunteers (9 males, 11 females, mean age  $54 \pm 16$  years) were studied as a control group. Except heparin during HD none of the patients or the healthy volunteers was receiving coagulation related medication. In particular none received antiplatelet agents or had been taking non-steroidal anti-inflammatory drugs for at least 20 days prior to the study. An informed consent was obtained from each individual enrolled into the study and the hospital ethics committee gave its approval to the study protocol.

### Methods

Blood samples for the assessment of platelet aggregation were drawn from the dialysis access site immediately before and shortly after the 2<sup>nd</sup> HD session of the week. Blood was then collected with citrate (1 part sodium citrate 3.8 %, 9 parts blood) in a polypropylene tube and centrifuged for 5 minutes at 500RCF in order to obtain platelet rich plasma (cPRP). The supernatant was transferred with a plastic pipette in a plastic test tube.

Platelet aggregation was evaluated by means of a slide platelet aggregation test kit (SPAT<sup>®</sup>; Analytical Control Systems, Fishers, IN, USA), which includes PG as platelet aggregation agonist. On a plastic microscope slide 100 $\mu$ l cPRP were mixed with 100  $\mu$ l SPAT reagent (Analytical Control Systems, Fishers, IN, USA). A stopwatch was started and the slide was rocked continuously until aggregation, in the form of many visible white clumps of platelets, occurred. The time needed for platelet aggregation to occur (platelet aggregation time) is as measure of platelet aggregability. All tests were done in duplicate at room temperature, and the mean value was obtained. One of the authors, blinded regarding the subjects' identity, performed all the tests in the same laboratory environment.

Concurrently, platelet count, hemoglobin (Hb), international normalized ratio (INR) and activated partial thromboplastin time (aPTT) were examined in blood samples obtained before the start of HD. Blood urea was assessed before the start and after the end of HD in order to estimate KT/V according to the second generation natural logarithmic formula, developed by Daugirdas based on the single pool urea kinetic model (36). The weekly dose of rHuEPO (units per Kg of patient's dry body weight) was registered for epoietin-alpha (Epo-a/bw) and darbepoietin-alpha (Dar-a/bw) and for both together (Epo/bw). Multiplying the dose of darbepoietin-alpha in micrograms by a factor of 200 gave its equivalent in weekly epoietin-alpha dose. Finally, the dose of tinzaparin per Kg of patient's dry body weight given in the examined hemodialysis session (LMWH/bw) was registered (Table 1).

**Table 1.** The values of factors that are evaluated for effect on platelet aggregation, expressed as mean $\pm$ SD

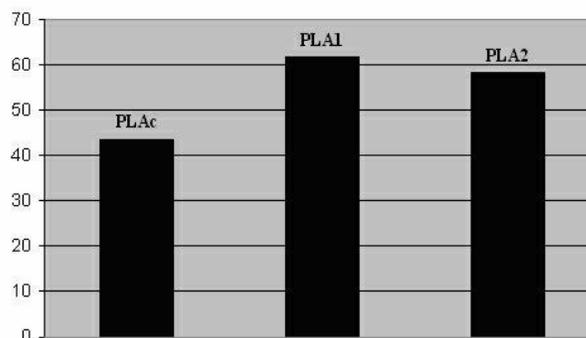
<b>platelet count (platelets/<math>\mu</math>l)</b>	249871 $\pm$ 66976
<b>Hb (g/dl)</b>	10.9 $\pm$ 1.15
<b>INR</b>	0.91 $\pm$ 0.09
<b>aPTT (patient/control sec)</b>	1.22 $\pm$ 0.75
<b>KT/V</b>	1.18 $\pm$ 0.44
<b>Epo/bw (units/Kg)</b>	189.64 $\pm$ 148.43
<b>Epo-a/bw (units/Kg)</b>	208.61 $\pm$ 139.88
<b>Dar-a/bw (<math>\mu</math>g/Kg)</b>	0.948 $\pm$ 0.802
<b>LMWH/bw (units/Kg)</b>	40.48 $\pm$ 19.47

### Statistical analysis

Results are expressed as a mean value and standard deviation (SD). Normality of the evaluated variables was assessed with the single sample Kolmogorov-Smirnov test. Unpaired t-test, paired t-test and Pearson correlation test were used where appropriate. P-values < 0.05 were considered as statistically significant.

### Results

Propyl gallate-induced platelet aggregation time, as it is assessed by the time needed for aggregation to occur, before the start of the HD session (PLA1) was significantly impaired in comparison with the control group (PLAc). PLA1 was  $62.74 \pm 15.80$ sec while PLAc was  $43.66 \pm 11.25$ sec ( $p < 0.0001$ ) (Figure 1).



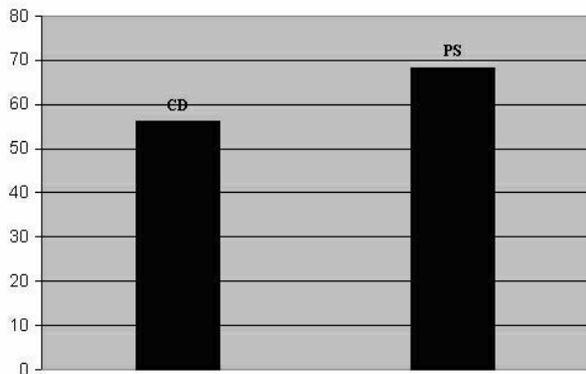
**Fig 1.** Platelet aggregation in HD patients and the effect of HD procedure

Platelet aggregation, as it is assessed by the time needed for aggregation to occur, before the start of the HD session (PLA1) was significantly impaired in comparison with the control group (PLAc). PLA1 was  $62.74 \pm 15.80$  sec while PLAc was  $43.66 \pm 11.25$  sec ( $p < 0.0001$ , unpaired t-test). Platelet aggregation after HD procedure (PLA2) was also significantly impaired in comparison with platelet aggregation in the control group (PLAc)  $58.59 \pm 18.67$  sec vs.  $43.66 \pm 11.25$  sec ( $p = 0.002$ , unpaired t-test). The hemodialysis procedure did not have a significant acute effect on platelet aggregation as PLA1 was  $62.74 \pm 15.80$  sec and PLA2 was  $58.59 \pm 18.67$  sec ( $p = \text{ns}$ , paired t-test).

Platelet aggregation after HD procedure (PLA2) was also significantly impaired in comparison with platelet aggregation in the control group (PLAc)  $58.59 \pm 18.67$  sec vs.  $43.66 \pm 11.25$  sec ( $p = 0.002$ ) (Figure 1).

The HD procedure did not have a significant acute effect on platelet aggregation as PLA1 was  $62.74 \pm 15.80$  sec and PLA2 was  $58.59 \pm 18.67$  sec ( $p = \text{ns}$ ) (Figure 1).

The type of the dialysis membrane used for treatment had an influence on platelet aggregation time assessed before the start of HD session ( $56.07 \pm 9.02$  sec for patients dialysed with CD membranes vs.  $68.23 \pm 18.19$  sec for patients using PS membranes;  $p = 0.023$ ) (Figure 2). However, the different dialysers had no acute effect on platelet aggregation, as the change in platelet aggregation time induced by HD (DPLA=PLA1-PLA2) did not differ significantly if CD membrane ( $2.5 \pm 11.88$  sec) or PS membrane ( $3.82 \pm 14.52$  sec) was used ( $p = \text{ns}$ ).



**Fig 2.** Long-term effect of dialysis membrane composition on platelet aggregation

Measurements taken before the start of HD session showed an effect of the type of dialysis membrane used on platelet aggregation ( $56.07 \pm 9.02$  sec for patients dialysed with CD membranes vs.  $68.23 \pm 18.19$  sec for patients using PS membranes ( $p = 0.023$ , unpaired t-test).

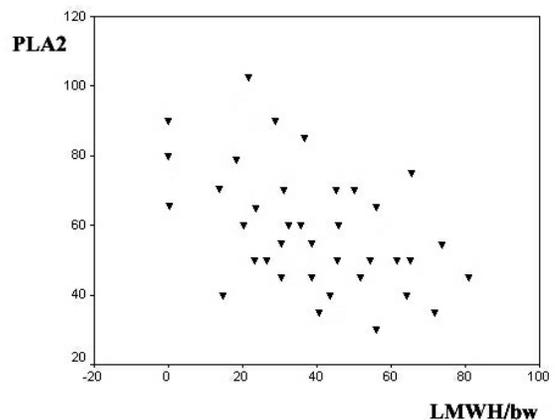
The type of dialysis access had no acute or chronic effect on platelet aggregation, since both DPLA and PLA1 in the A-V fistula group ( $0.25 \pm 11.97$  sec and  $59.50 \pm 12.12$  sec respectively) did not differ significantly from DPLA and PLA1 in the central intravenous catheter group ( $8.63 \pm 14.15$  sec and  $68.63 \pm 20.25$  sec respectively) ( $p = \text{ns}$ ).

Dialysis adequacy had no effect on platelet aggregation, since no correlation was found between KT/V and the platelet

aggregation time before HD ( $r = 0.113$ ,  $p = 0.277$ ) or after HD ( $r = 0.250$ ,  $p = 0.087$ ).

Furthermore, platelet aggregation was not correlated with the weekly dose of rHuEPO, calculated as units/kg of dry body weight (Epo/bw) ( $r = 0.063$ ,  $p = 0.368$ ). Results were similar regardless of the type of rHuEPO used; either epoietin-alpha (Epo-a/bw) ( $r = 0.019$ ,  $p = 0.468$ ) or darbepoietin-alpha (Dar-a/bw) ( $r = 0.228$ ,  $p = 0.278$ ) separately. The type of rHuEPO administered, had no significant effect on platelet aggregation time (epoietin-alpha:  $59.5 \pm 9.16$  sec vs. darbepoietin-alpha:  $70.00 \pm 24.74$  sec,  $p = 0.248$ ).

A statistically significant negative correlation was almost reached between the dose of tinzaparin sodium, calculated as units/kg of dry body weight (LMWH/bw) and platelet aggregation time as measured before the start of the hemodialysis session - PLA1 ( $r = -0.332$ ,  $p = 0.056$ ). This negative correlation became much stronger and statistically significant after the HD procedure ( $r = -0.428$ ,  $p = 0.009$ ) (Figure 3).



**Fig 3.** The effect of tinzaparin administration on platelet aggregation

The time needed for PLA aggregation to occur after the end of the HD procedure was strongly correlated with the dose of tinzaparin sodium per Kgr of dry body weight administered. The higher the dose of tinzaparin administration, the less the time needed for PLA aggregation after the end of the HD procedure. ( $r = -0.428$ ,  $p = 0.009$ , Pearson correlation test)

As expected, platelet aggregation time was negatively correlated with whole blood platelet count ( $r = -0.563$ ,  $p = 0.001$ ). On the other hand, platelet aggregation time was not correlated with Haemoglobin (Hb) values ( $r = -0.056$ ,  $p = 0.386$ ), INR ( $r = -0.162$ ,  $p = 0.308$ ) or aPTT ( $r = -0.089$ ,  $p = 0.386$ ).

## Discussion

The simple and inexpensive PG induced platelet slide aggregometry test (32) was used in this study in order to evaluate platelet aggregation in HD patients. Propyl-gallate was chosen because of its unique properties. Clinical studies demonstrated that PG is possibly the most suitable platelet aggregation agonist for evaluating the effect of aspirin and non-steroid-antiflammatory-drugs (NSAIDs) on platelet aggregation, indicating that this compound is the proper reagent for detection of impaired platelet arachidonate metabolism (37,38). As mentioned above, impaired platelet

arachidonate metabolism is incriminated for defective platelet aggregation in HD patients. Recently, Xiao et al demonstrated that PG-induced platelet aggregation and tyrosine phosphorylation of multiple proteins were substantially abolished by aspirin, apyrase, and abciximab, suggesting that PG is associated with activation of platelet cyclooxygenase 1, adenosine phosphate receptors, and GpIIb/IIIa, respectively (34). Abnormal function of all these factors has been implicated in platelet dysfunction in HD patients (3-6). Propyl-gallate is possibly a suitable reagent for a global evaluation of platelet aggregation in HD patients, in contrast with other commonly used aggregation reagents like arachidonate, ADP, epinephrine, fibrinogen and others, which are proper for specific, but only partial evaluation of platelet aggregation (35).

As expected, impaired platelet aggregation was detected in HD patients. Interestingly, platelet aggregation impairment did not show any improvement after the HD procedure. This is in contrast with other studies that detected enhancement of platelet aggregation after the HD procedure (3,26). On the other hand, there are studies, which indicated that HD procedure reduces platelet aggregation (27,28). The difference among these studies could be attributed to the different HD conditions (i.e. HD membrane composition or type of anticoagulant) or to the different platelet aggregation agonists used. In our study PG was used for the first time in order to evaluate platelet aggregation in HD patients. As already noted, PG is a platelet agonist that can detect impairments in many pathways leading to platelet aggregation. Consequently, our study assessed platelet aggregation more globally than others, which used more specific platelet aggregation agonists that are capable for evaluating only one pathway of platelet aggregation each (35).

Regarding the HD procedure per se, we failed to detect any difference. Type of dialysis access (A-V fistula or central intravenous catheter) did not affect platelet aggregation. Two kinds of dialysis membranes were used in our study, CD and PS. There are many studies that evaluate the effect of membrane composition on platelet activation or aggregation. Most of them evaluated the fibrinogen binding to GpIIb/IIIa receptor but the results are contradicting. In one study the level of bound GPIIb/IIIa fibrinogen receptor was increased when PS membrane was used but there was no significant change with cellulose triacetate membrane (29). In contrast, another study indicated that the degree of GPIIb/IIIa activation was greater during hemodialysis with regenerated cellulose than PS membrane (30). In a paper comparing the influence of cuprophan, hemophan, and PS on fibrinogen binding on platelets, Gawaz et al concluded that it is increased only when cuprophan membrane was used (31). Finally, another study comparing polymethylmethacrylate, cuprophan, and PS membranes did not demonstrate any effect of the type of dialysis membrane on platelet aggregation (27). In our study, we detected that PG induced platelet aggregation time before HD session was higher when PS membrane was used in comparison with CD membrane. That was a long-term effect since there was not difference in the change in platelet aggregation time before and after HD if PS or CD dialysers were used. The fact that PG induced platelet aggregation is totally abolished by the GpIIb/IIIa inhibitor abciximab in relatively low concentrations (34), in combination with the results of the above studies, raises the

possibility that PS membrane induces less GpIIb/IIIa activation than CD membrane.

Dialysis adequacy (as expressed by KT/V) did not have any effect on platelet aggregation either. This means that the clearance of the low molecular weight toxins by HD does not influence platelet aggregation. On the other hand, the role of some low molecular weight toxins, especially of guanidinosuccinic acid, in uremic platelet dysfunction is well established (7). It is possible that the difference in urea clearances, among our patients was not big enough in order to induce difference in platelet aggregation. Standard HD, 3 times a week, 4 hours per session, seems adequate in removing enough guanidinosuccinic acid or other low molecular weight uremic toxins, responsible for platelet dysfunction.

Nowadays rHuEPO is administered in most HD patients. The beneficial effect of rHuEPO on hemostasis is well established. One certain mechanism is improvement in blood rheology due to correction of anemia (9). Many studies, using various platelet agonists, have evaluated the effects of rHuEPO on platelet aggregation in HD patients. Again the results were conflicted, as some authors detected an increase in platelet aggregation after rHuEPO administration (10-14), while others failed to do so (9,15-17). In our study we did not detect any correlation between platelet aggregation time and the weekly dose of rHuEPO per Kg of dry body weight (Epo/bw). There was no statistically significant difference in platelet aggregation if epoietin-alpha or darbepoietin-alpha was administered. It is notable that in another study platelet aggregation responses to thrombin did not differ between the two treatments (39).

In most HD patients, extracorporeal blood circulation is maintained with the administration of unfractionated heparin, or more recently with the administration of LMWH. Studies in non-uremic subjects indicated that unfractionated heparin activates platelets and increases platelet aggregation. In non-uremic subjects LMWH also seems to increase platelet aggregation, although to a lesser extent than unfractionated heparin (22-25). There are some studies in HD patients that confirm the platelet aggregation enhancement by unfractionated heparin (18,19). On the other hand, there are quite a few studies that evaluate the effect of the more recently used LMWH on platelet aggregation in HD patients. In one study LMWH did not alter ADP or collagen induced platelet aggregation during dialysis (20), and in another one no differences between unfractionated heparin and LMWH were observed regarding the platelet aggregation in HD patients (21). In our unit the major anticoagulant used was the LMWH tinzaparin sodium. Platelet aggregation before HD was positively, although weakly, correlated with the dose of tinzaparin sodium (units/kg of dry body weight). This positive correlation became much stronger after HD, i.e. near tinzaparin administration, indicating a short-term effect of that LMWH on platelet aggregation. The positive impact of LMWH on platelet aggregation could be the result of the less microthrombus formation and consequently of the less fibrinolytic mechanism activation and fibrinogen fragments production during HD procedure. According to a study by Sreedhara et al, platelet dysfunction in chronic HD patients results from decreased GpIIb/IIIa availability due to receptor occupancy by fibrinogen fragments (40). Additionally, Sobel et al have detected that unfractionated heparin directly binds

to GpIIb/IIIa modulating its function (41). This aspect has not been evaluated yet for LMWH.

Finally, no correlation was detected in our study among platelet aggregation and Hb, INR or aPTT, indicating, as expected, that PG induced platelet aggregation is independent of other factors that are correlated with blood rheology or coagulation. In contrast, also as expected, PG induced platelet aggregation was positively correlated with the whole blood platelet count.

## Conclusions

In conclusion, our study demonstrates that PG induced platelet aggregation in chronic HD patients is decreased in comparison with non-uremic subjects. The HD procedure does not influence platelet aggregation neither does the dialysis access type (A-V fistula or central intravenous catheter) or the low molecular weight uremic toxins clearance. Different types of dialysis membranes have different effects on platelet aggregation. Polysulfone dialyser prolongs the PG-induced platelet aggregation time in comparison to CD dialyser. Finally, from the medications usually administered in HD patients, only LMWH had a positive effect on PG-induced platelet aggregation. The clinical significance of the above results, in relation with the bleeding or thrombotic episodes in HD patients, needs further evaluation.

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