Does Pycnogenol Intensify the Efficacy of Acetylsalicylic Acid in the Inhibition of Platelet Function? *In vitro* experience

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Introduction: Some compounds of herbal origin, such as Pycnogenol® (PYC), have been considered as an aid in antiplatelet therapy. Pycnogenol®, a French maritime pine bark extract, is a complex mixture of polyphenols that has the ability to reduce human smoking-induced platelet aggregation. The aim of this study was to evaluate the *in vitro* ability of PYC to improve the efficacy of acetylsalicylic acid (ASA) in the inhibition of platelet function.

Materials/Methods: Whole blood, anticoagulated with hirudin, was drawn from 38 volunteers (40.4±13.8 years old) and incubated with PYC (10, 50, 100 µg/ml) or/and ASA (25, 100 µmol/l) for 20 min at RT.

PYC was dissolved in water (water-PYC group, n=20) or ethanol (ethanol-PYC group, n=18). To investigate platelet functions, PFA-100™ closure-time determination, whole-blood electrical aggregation (WBEA), and PRP aggregation were employed. Collagen (1 µg/ml) and ADP (5 µmol/l) were used as platelet agonists.

Results: A compounding effect of ASA and PYC to inhibit platelet function recorded in collagen-induced aggregation in PRP was observed, but only when ethanol-dissolved PYC was used. The inhibitory effect of PYC (alone) was most profound in platelets activated with ADP. At all concentrations, PYC significantly inhibited platelet aggregation only in the ethanol-PYC group.

Discussion: It was found that under *in vitro* conditions, ethanol-dissolved PYC deepened the efficacy of ASA to inhibit platelet function. This study confirmed the direct and compounding (with ASA) inhibitory effect of PYC on platelets. These observations encourage the concept that the combined use of ASA and PYC may be beneficial in patients with impaired response to ASA therapy.

Key words: Pycnogenol • Acetylsalicylic Acid (ASA) • platelet inhibition

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Abbreviations: A_{max} – the extent of maximal platelet aggregation; **ASA** – acetylsalicylic acid; **IPI** – index of platelet inhibition; CT_{CEPI} – closure time in the collagen epinephrine cartridge; CT_{CADP} – closure time in the collagen ADP cartridge; **PRP** – platelet-rich plasma; **PFA** – platelet function analyzer; **PYC** – Pycnogenol; **RT** – room temperature; **WB** – whole blood; **WBEA** – whole-blood electrical aggregometry.

Introduction

Commonly used antiplatelet agents (acetylsalicylic acid, thienopyridine derivatives) have been proven effective in vascular diseases [20,26]. In some patients, however, an impaired response to antiplatelet drugs can be observed [8,10,11]. On the other hand, polyphenolic compounds of herbal origin have been reported to modestly reduce the risk of cardiovascular disease [12,28] and to have inhibitory effects on platelet function [3,7,24]. Polyphenol-rich red grapes, cocoa, and pine bark extracts have been shown to exert positive effects on vascular function and to influence (inhibit) platelet function in humans [7]. The majority of studies concerning the effects of polyphenols and phenolic plant extracts on platelet functions were carried out *in vitro* [14,22,23].

Some compounds of herbal origin, such as Pycnogenol® (PYC), have been considered as an aid in antiplatelet therapy [15,21]. PYC is a standardized extract of the bark of French marine pine. It is a complex mixture of phenolic acids and procyanidins (biopolymers containing catechin and epicatechin) [25].

It was reported that the effectiveness of the combined action of low-dose PYC and ASA in the inhibition of platelet aggregation induced by smoking was comparable to the effect of aspirin alone used at a five-fold higher dose [22]. Regardless of occasional clinical investigations on PYC, its effects on platelet function and reactivity have not been adequately discussed in the literature so far. There are only two literature reports on the modulation of ASA-induced inhibition of platelet aggregation by polyphenolic compounds [21,22]. We have no information about the combined effects of PYC and ASA on blood platelets. Accordingly, the aim of this study was to evaluate the ability of PYC to improve the efficacy of acetylsalicylic acid (ASA) in the inhibition of platelet functions monitored under *in vitro* conditions.

Materials and Methods

Reagents

Hirudin (Refludan®, lepirudin (rDNA) for injection) was purchased from Aventis (Aventis Pharma Deutschland GmbH, Bad Soden a. Ts, Germany) and Laspal® (lysine acetylsalicylate), was obtained from Synthelabo (Synthelabo Groupe, Quetigny, France). The platelet agonists: collagen, ADP and arachidonic acid (AA) were provided by Chrono-Log (Havertown, PA, USA). Pycnogenol® (PYC) (Horphag Research) is the trade name of a "standardized extract of the bark of the French marine pine (Pinus pinaster, Ailton subsp. atlantica des Villar)" and was obtained from the Drug Research Institute, Modra, SR.

Unless stated otherwise, all other chemicals were from Polish Chemical Reagents (Gliwice, Poland).

Blood donors

Blood anticoagulated with hirudin (50 µg/ml) was drawn from 38 healthy volunteers (40.4±13.8 years of age). Two approaches were used in this study:
(a) PYC dissolved in deionized water (water-PYC group, incl. 20 healthy volunteers),
(b) PYC dissolved in 10% ethanol (ethanol-PYC group, incl. 18 healthy volunteers).

The study was performed under the guidelines of the Helsinki Declaration for human research and approved by the committee on the Ethics of Research in Human Experimentation at the Medical University of Łódź (No RNN/21/04/KE).

Measurements of platelet reactivity

The whole blood or platelet-rich plasma (PRP) was incubated with PYC (10, 50, 100 µg/ml) or/and ASA or appropriate vehicle for 20 min at RT. The concentrations of ASA were established individually for each donor to achieve the inhibition of platelet reactivity of at least 25% in the range of 25 µmol/l to 100 µmol/l ASA.

Platelet reactivity was estimated using two aggregometry methods: optical and whole blood electrical aggregometry. For both aggregation methods the aggregation curves were analyzed using the software "Platelet Aggregation Monitoring and Analysis" (PAMA) [8].

Optical platelet aggregation measurements

PRP was obtained by centrifugation and platelet count was adjusted to $3.0×10^8$ platelets per ml. PYC was added to PRP, incubated for 15 min at RT, preincubated for 5 min at 37°C, and then supplemented with ADP (final concentration: 5 µmol/l) or collagen (1 µg/ml). Platelet aggregation was monitored for 15 min according to the manufacturer's protocol using the optical aggregometer Chrono-Log 490-2D (Chrono-Log, Havertown, PA, USA). The value of maximal light transmission, A_{max}, reflecting the extent of maximal platelet aggregation, was used for further data analysis.

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Whole blood electrical aggregometry (WBEA)

Aggregation was studied in whole blood by an impedance technique using a Whole Blood Aggregometer Chrono-Log 592 (Chrono-Log, Havertown, PA USA), and the measurements were performed according to the Chrono-Log protocol [2].

Pycnogenol was added to whole blood and incubated for 15 min at RT. After that the blood sample (0.5 ml) was diluted 1:1 with 0.85% saline, preincubated for 5 min at 37°C, then supplemented with either ADP (final concentration: 5 µmol/l) or collagen (1 µg/ml) and platelet aggregation was monitored for 15 min. The value of impedance (Ω), reflecting the extent of maximal platelet aggregation (A_{max}) was used for further data analysis.

PFA-100™ studies

The flow analyzer PFA-100™ (Dade, Miami, FL, USA) allows studying blood platelet reactivity under dynamic conditions. All blood samples were tested for closure times with CEPI (CT_{CEPI}) and CADP (CT_{CADP}) cartridges according to the manufacturer's instructions no earlier than 30 min after and no later than 1 h after blood drawing. Since the antiplatelet action of aspirin manifests as a prolonged CT_{CEPI} and unchanged CT_{CADP}, the determination of CT_{CEPI} was used as a marker of platelet responsiveness to aspirin, whereas CT_{CADP} was employed to detect any other possible dysfunctions in the initial platelet reactivity. PFA-100™ measurements were performed in duplicate and averaged CT values were used for further data analysis [16,17].

The method of presenting platelet aggregation results

To evaluate the extent of inhibition of platelet function by ASA, PYC, and PYC plus ASA in our study, we calculated the differences between the observed values of aggregation in PRP, WBEA aggregation, and PFA-100™ closure times and the average values corresponding to the reference blood samples (not treated with PYC or ASA). Such differences, standardized to SD values, were further used to calculate a numeric score, the Platelet Inhibition Index (IPI), using special formulas reported and discussed earlier [8,29].

For ADP-induced aggregation in PRP and WBEA

$$\mathbf{IPI}_{ADP} = [\text{Std (x/SD): } A_{max} - A_{max(average\ reference)}]_{PRP} + [\text{Std (x/SD): } A_{max} - A_{max(average\ reference)}]_{WBEA}$$

For determinations of platelet reactivity in whole blood (PFA and WBEA collagen-induced aggregation):

$$\mathbf{IPI}_{WB} = [\text{Std (x/SD): } CT_{CEPI(average\ reference)} - CT_{CEPI}]_{PFA} + [\text{Std (x/SD): } A_{max} - A_{max(average\ reference)}]_{WBEA\ collagen}$$

For collagen-induced platelet aggregation in PRP:

$$\mathbf{IPI}_{PRP} = [\text{Std (x/SD): } A_{max} - A_{max(average\ reference)}]_{collagen}$$

where $[A_{max} - A_{max(average\ reference)}]$ and $[CT_{(average\ reference)} - CT_{CEPI}]$ are the calculated differences between the observed values and the averaged reference values for the given methods, and Std (x/SD) denotes the standardization of such differences to a within-group SD.

Application of **IPI** enables us to summarize the effects of an investigated compound monitored with different methods of platelet reactivity.

In the case of collagen-induced aggregation in PRP, IPI shows the extent of platelet inhibition for one method used for monitoring platelet reactivity.

Statistical analysis

Medians and interquartile ranges (*Me*, *IQ*) are given for all parameters showing departures from normality (according to Shapiro-Wilk W test), and the results were analyzed using the non-parametric Kruskal-Wallis test and the post hoc Connover-Inman test for multiple comparisons.

RESULTS

Impact of the solvent on the efficacy of Pycnogenol in platelet function inhibition

In PYC dissolved in water we detected a slight inhibitory effect, while PYC dissolved in 10% ethanol showed a potent effect. The most significant differences were revealed in the case of PRP aggregation with collagen as an agonist. In the presence of PYC dissolved in water or ethanol, the platelets responded to ASA as follows:

$IPI_{PRP} = -1.83$ (−2.66; −0.92) for water vs.
$IPI_{PRP} = -11.08$ (−11.65; −10.21) for ethanol (Figure 1).

The inhibitory effects of PYC alone were most profoundly expressed in the ADP-stimulated platelet aggregation. PYC, at all the concentrations tested, significantly decreased platelet aggregation only in the case of ethanol-PYC (Figure 2).

Effect of PYC on platelet reactivity

At all the concentrations tested, PYC alone significantly blocked ADP-dependent platelet aggregation only when dissolved in ethanol (see above). Comprehensive measures of IPI_{ADP} for both WBEA and PRP aggregation are presented in Figure 2.

In collagen-induced PRP aggregation, PYC dissolved in ethanol was an effective inhibitor of platelet function only at the higher concentration of 100 µg/ml: IPI = −1.19 (−2.64; −0.75) vs. 0.59 (−0.73; 0.82) in the control.

We also detected a slight inhibitory effect of PYC on platelet reactivity recorded in whole blood with the use of PFA-100™ CT_{CEPI} and in the collagen-induced aggregation in whole blood (Table 1). No effect on platelet reactivity of the low PYC concentration of 10 µg/ml was revealed in any applied method.

The ability of PYC to improve the efficacy of ASA in the inhibition of platelet function

PRP measurements

We observed the compounding effect of PYC to ASA-mediated inhibition of platelet function, recorded in collagen-
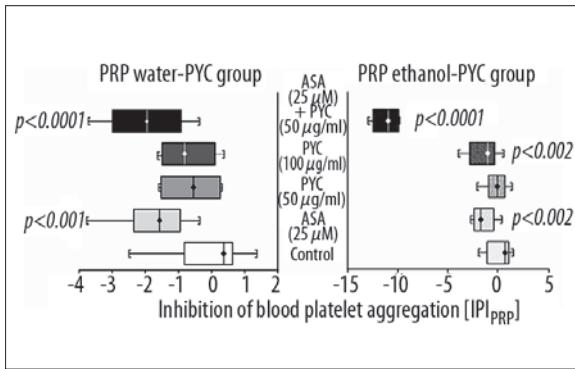
Figure 1. The compounding effect PYC on ASA-mediated inhibition of collagen-induced platelet aggregation in PRP. Data represent the Index of Platelet Inhibition (IPI), estimated as described in 'Materials and methods'. Significance of the difference between water-PYC + ASA and ethanol PYC + ASA: $p<0.05$. Data shown as median, min-max (whiskers), and interquartile range (box)
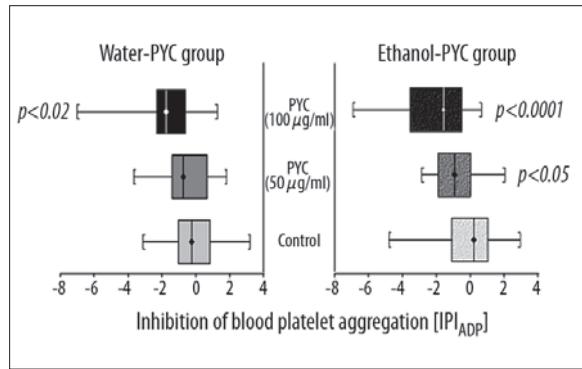
Figure 2. The inhibitory effect of PYC on platelet aggregation (WBEA and PRP) stimulated with ADP (5 µM). Data represent the Index of Platelet Inhibition (IPI), estimated as described in 'Materials and methods'. Significance of the difference in platelet inhibition between water-PYC + ASA and ethanol PYC + ASA: $p<0.02$. Data shown as median, min-max (whiskers), and interquartile range (box)

Table 1. The effect of ASA, PYC, and ASA + PYC on platelet reactivity in whole-blood measurements. The values of the Index of Platelet Inhibition (IPI) represent the overall measure of platelet reactivity monitored by PFA-100™ closure time and collagen-induced (1 µg/ml) platelet aggregation (WBEA) for water-PYC and ethanol-PYC. Data shown as median and interquartile range

Blood platelet reactivity/ response to aspirin and/or pycnogenol	IPI ASA (50 µM)	IPI PYC (50 µg/ml)	IPI PYC (100 µg/ml)	IPI PYC (50 µg/ml) and ASA
Water-PYC (n=20)	**−2.33** (−3.51; −1.05)	**−0.17** (−0.55; 0.56)	**−0.05** (−1.65; 0.48)	**−2,42** (−3.77; −0.48)
Ethanol-PYC (n=18)	**−1.63** (−2.88; −0.42)	**0.06** (−1.94; 0.55)	**−0.49** (−1.00; 0.42)	**−1.84** (−2.05; −1.02)

induced PRP aggregation, but only in the ethanol-dissolved PYC group (Figure 1).

The inhibition of platelet aggregation, estimated as IPI_{PRP} and expressed as medians and interquartile ranges (*Me, IQ*), was most profound for the concentrations of 50 µg/ml PYC and 25 µmol/l ASA: $IPI_{PRP} = -11.08$ (−11.65; −10.21) vs. $IPI_{PRP} = -1.82$ (−2.40; −0.47) for ASA alone.

Whole-blood measurements

Our data present a very slight intensifying effect of PYC on ASA-induced platelet inhibition recorded with the use of PFA-100™ CT_{CEPI} and collagen-induced aggregation in a whole blood (WBEA).

Table 1 shows the medians of the inhibition rates examined with the application of CT_{CEPI} measurements (PFA-100™) and whole-blood electrical aggregometry with collagen as agonist. We observed a significant inhibitory effect of ASA and a slight compounding effect of PYC.

Discussion

Our study evaluated for the first time the *in vitro* ability of PYC to increase the efficacy of acetylsalicylic acid (ASA) in the inhibition of platelet function. Our observations may

be considered an argument for designing clinical trials with PYC as an aid in antiplatelet therapy in patients with "aspirin resistance". There is evidence that antiplatelet therapy reduces cardiovascular disease risk; however, an impaired response to ASA therapy may be considered a real problem [5,8,10]. Our current *in vitro* study encourages the simultaneous use of ASA and PYC.

Putter et al. reported that the consumption of PYC reduced smoking-induced platelet aggregation [22]. Pearson et al. described a synergic inhibitory effect of flavonoid-rich cocoa and ASA on platelet functions [21]. In general, the polyphenol-rich substances from red grapes, cocoa, and pine bark extracts have been shown to exert positive effects on vascular function and to inhibit platelet function [1,7,24]. When we take into consideration the *in vitro* effects of polyphenols and phenolic plant extracts on platelets function [14,23], it seems tempting and justified to evaluate the *in vitro* ability of PYC to improve the efficacy of ASA in the inhibition of the platelet functions.

In the current study we observed a significant inhibitory effect of PYC alone and a slight compounding effect of PYC in the presence of ASA. Whereas the outcomes of the studies accomplished hitherto indicated that PYC might increase the efficacy of ASA in inhibiting platelet function monitored in PRP, our results point out that PYC

is also able to improve the efficacy of ASA in the inhibition of platelet functions in a more natural platelet milieu, i.e. in whole blood.

Pycnogenol is known to stimulate constitutive endothelial NOS (eNOS) activity and it is able to increase NO generation and decrease both platelet aggregation and adhesion [6]. Theoretically it is conceivable to take into consideration the mechanism of PYC action on platelets through platelet NOS stimulation, thereby modifying the PYC-mediated platelet response to ASA. On the other hand, PYC and other polyphenols show antioxidant activity and may protect platelets from peroxidative stress and thus suppress their proaggregatory effect [18,25].

A variety of molecules present in the diet have been shown to inhibit platelet activation; however, the detailed mechanisms of antiplatelet function of plant-derived polyphenol-rich extracts have not yet been established. Relevant to the topic of our study are reports indicating that the direct effects of flavonoids on platelet inhibition may be dependent on the cyclooxygenase pathway [9,14,19,27]. These observations may contribute to the explanation of PYC's ability to improve the efficacy of ASA in the inhibition of platelet function.

Our *in vitro* study revealed a potent capability of PYC alone to inhibit platelet aggregation with ADP. In the light of our present observations, the interaction of flavones and other phytochemicals with platelet adenosine receptors may also be considered a possible mechanism of the inhibition of platelet function. Genistein (present in a soy) was found to bind to A1 receptors [13]. Hence, adenosine receptor antagonism may be important in the spectrum of biological activities reported for flavonoids. Thus we provide a novel argument to support the importance of plant-derived polyphenols in the modulation of ADP-induced platelet aggregation.

This study describes the action of a complex mixture composed of numerous compounds, such as phenolic acids and procyanidins [23,25]. Pycnogenol contains both hydrophilic and lipophilic components. In our previous work we found that the lipophilic components present in PYC exhibit 6.2 times higher antioxidant ability than the hydrophilic components [4]. There is an opinion that due to such versatility, the Pycnogenol mixture may be able to prevent vascular diseases more effectively than single compounds.

Even though these results do not provide convincing evidence of the mechanisms of a direct antiplatelet action of PYC or explore the mechanisms of ASA–PYC interaction(s), they certainly encourage further investigation of this problem.

The results of our study support the concept presented in clinical studies on the usage of polyphenol derivatives as an aid in antiplatelet therapy. They suggest that a combination of antiplatelet drugs with preparations of herbal origin (food supplements) [15,21,22] may be beneficial in some clinical states.

Our current *in vitro* study confirmed the direct and compounding inhibitory effect of flavonoids on platelets inhibited with ASA, previously described in clinical investigations. PYC might by considered as the source for a new generation of more effective herbal "antiplatelet drugs". Our observations encourage the idea of the combined use of ASA and PYC in patients with impaired response to ASA. Although the effects of PYC on platelet aggregation are not enormous, they may be significant in compounding and/or correcting an incomplete therapeutic effect of ASA.

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