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Letter to the Editors-in-Chief

Effect of rivaroxaban and dabigatran on platelet functions: in vitro study

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ABSTRACT

Introduction: Clinical benefit-risk balance of direct oral anticoagulants (DOAC) in atherothrombosis prevention differs between anti-Xa and anti-IIa drugs and their specific effect on platelet functions remains controversial. We hence investigated rivaroxaban and dabigatran effect on platelets in identical experimental conditions.

Materials and methods: Blood of fifteen healthy volunteers was spiked with DOAC which plasma concentrations were measured by specific anti-Xa or anti-IIa assays. Light transmission aggregometry measured in platelet-rich plasma used low doses of agonists: 0.5 mM arachidonic acid, 2.5 μM ADP, 0.5 μM epinephrine, 0.8 μg/ml collagen, 7.5 μM TRAP-6 and 0.5 pM tissue factor in the presence of H-Gly-Pro-Arg-Pro-OH to prevent fibrin polymerization. Platelet adhesion on collagen fibres was evaluated in whole blood under flow. Same experiments were reproduced in the presence of aspirin.

Results: Median [95% CI] plasma concentrations were of 28 [23–36], 128 [119–144] and 321 [293–361] ng/ml for rivaroxaban and 39 [34–45], 171 [166–193] and 353 [349–382] ng/ml for dabigatran. DOAC did not modify platelet aggregation or adhesion on collagen fibres at any tested concentrations. However, they delayed platelet aggregation secondary to coagulation activation with a more potent effect with dabigatran ($p < 0.001$). Aspirin did not modify DOAC effect.

Conclusion: Efficacy of combining DOAC and aspirin in atherothrombosis prevention would not stem from a direct antiplatelet effect of the formers but to their additive inhibitory effect on platelet aggregation secondary to coagulation activation. This effect differs according to DOAC molecules and may also result from the pleiotropic roles of the different coagulation factors targeted by DOAC.

1. Introduction

Rivaroxaban is the unique Direct Oral AntiCoagulant (DOAC) authorized in Europe in combination with antiplatelet therapy for secondary prevention of atherothrombotic events after acute coronary syndrome [1]. Moreover, Eikelboom et al. showed in the COMPASS trial including patients with stable coronary or peripheral artery disease that rivaroxaban in combination with aspirin presented a net clinical benefit balance compared to aspirin alone, taking into account cardiovascular death, stroke, myocardial infarction events together with fatal or symptomatic bleeding into critical organ [2]. Concerning dabigatran, some clinical studies showed either no reduction in thrombotic events or increased major bleeding compared with placebo [3,4] while another reported significant reduction of cardiovascular mortality and thromboembolic risk [5]. Therefore, the net clinical benefit remains to be established for every DOAC molecule in each clinical setting.

Mechanism by which the DOAC-aspirin regimen reduces atherothrombotic risk compared with aspirin is uncertain. While the simplest explanation is that combining anticoagulant to antiplatelet agent would result in superior antithrombotic activity, a potential direct effect of DOAC on platelet functions should be re-evaluated. Indeed being FXa or thrombin inhibitor, DOAC could have different effects on platelet function. FXa acts upstream of thrombin in the coagulation cascade. Besides, it activates protease-activated receptor (PAR)-2 which is absent on platelet membrane, whereas thrombin is a potent activator of human platelets through PAR-1 and PAR-4. Consequently, these two factors are not equal with regard to platelet activation, neither might be their inhibitors.

We hence evaluated and compared the effect of rivaroxaban and dabigatran on platelet functions in strictly identical experimental conditions.

2. Materials and methods

The study was conducted in accordance with the ethical principles of the Declaration of Helsinki. Fifteen healthy fasting volunteers (male/female, 8/7; age, 35 ± 15 years) were included after informed consent.

Rivaroxaban (Bayer Healthcare AG, Germany), dabigatran (SelleckChem, Euromedex, France) or their vehicle (saline buffer containing 1% dimethyl sulfoxide, DMSO) were added to whole blood samples, incubated for 30 min at room temperature then centrifuged to obtain platelet rich (PRP) or poor (PPP) plasmas. Aspirin (Sanofi-Aventis, France) or its vehicle (demineralized water) was added to PRP at 100 μg/ml final concentration as previously described [6]. DOAC concentrations were measured in PPP by specific anti-Xa (STA-Liquid

Abbreviations: ADP, adenosine diphosphate; DMSO, dimethyl sulfoxide; DOAC, direct oral anticoagulants; F, factor; GPRP, H-Gly-Pro-Arg-Pro-OH; PAR, protease activated receptor; PBS, phosphate buffered saline; PPP, platelet poor plasma; PRP, platelet rich plasma; TRAP, thrombin receptor activating peptide; VKA, vitamin K antagonists

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anti-Xa; Stago, France) or anti-IIa assays (Hemoclot Thrombin Inhibitor, Hyphen Biomed, France), respectively.

Platelet aggregations were measured in PRP by light transmission aggregometry on a ThromboAggregometer 8 V. They were induced by agonists (Stago) at low concentrations to detect any potential pro- or anti-aggregant effect of DOAC: ADP (2.5 μM), arachidonic acid (0.5 mg/ml), epinephrine (0.5 μM), collagen (0.8 μg/ml) and Thrombin Receptor-1 Activating Peptide 6 (TRAP-6) (7.5 μM). Platelet agglutination was induced by ristocetin (1.2 mg/ml). Platelet aggregation was also induced following coagulation activation in PRP with 0.5 pM recombinant human tissue factor (Innovin, Dade Behring, Germany) and 10 mM $CaCl_2$, in the presence of 2 mg/ml H-Gly-Pro-Arg-Pro-OH (GPRP; Pefabloc FG, Cryopep, France) to prevent fibrin polymerization. Three parameters were analysed: maximum amplitude of platelet aggregation (%) normalised to control PRP of each volunteer, lag time (*sec*) and velocity (%/sec).

Platelet adhesion to fibrillar collagen (50 μg/ml) was studied in DOAC spiked whole blood from 3 healthy volunteers. Platelets were stained with calcein AM (1 μg/ml, ThermoFischer Scientific, USA) for 30 min at room temperature in the dark. A shear rate of 1200/s was used to mimic arterial shear conditions in vitro. Mean surface coverage and mean fluorescence intensity were analysed at 1, 3 and 5 min of flow.

Results were expressed as mean ± SD or median [95% confidence interval]. Friedman test on matched data followed by Dunn tests for pair-wise comparisons were performed. Statistical significance was accepted when *p*-value was below 0.05.

3. Results

DOAC median [95% CI] concentrations in PPP were of 28 [23–36], 128 [119–144] and 321 [293–361] ng/ml for rivaroxaban and 39 [34–45], 171 [166–193] and 353 [349–382] ng/ml for dabigatran. DOAC did not modify platelet aggregation parameters (Table 1; $n = 12$) or profiles (reversibility, number of phases and platelet shape change) compared to control PRP whatever the concentrations tested.

Moreover, despite the small sample size of the platelet adhesion analyses ($n = 3$), our study suggested that neither rivaroxaban nor dabigatran modified platelet adhesion to collagen fibres or aggregate formation as neither the mean fluorescence intensity nor the mean surface coverage differed after 1, 3 or 5 min of flow compared to control (data not shown).

Thrombin generation was thereafter triggered in PRP ($n = 12$) with tissue factor in the presence of GPRP. Thrombin thus generated induced platelet aggregation without fibrin clot formation. A concentration-dependent delay of this aggregation was observed in the presence of DOAC (Fig. 1A). The effect was more significant with dabigatran than with rivaroxaban in terms of lag time lengthening ($p < 0.05$). Moreover, only dabigatran decreased the velocity at a concentration higher than 171 ng/ml ($p < 0.05$) (Fig. 1B). Once activated, platelets eventually aggregated and the maximum amplitude remained unchanged even at high DOAC concentrations (Fig. 1C).

Aspirin did not modify the formerly observed DOAC effect on platelet aggregation parameters following coagulation activation (Fig. 1D, E, F; $n = 6$) whereas its specific inhibitory effect on platelet thromboxane generation was checked by a complete inhibition of arachidonic acid-induced platelet aggregation (data not shown).

4. Discussion

Here, we demonstrated that neither rivaroxaban nor dabigatran modified platelet aggregation induced by low dose standard agonists or platelet adhesion under arterial flow conditions. Nevertheless, by inhibiting thrombin generation following coagulation activation, they delayed platelet aggregation secondary to coagulation activation with a more potent effect of dabigatran compared to rivaroxaban. The clinical impact of this differential effect remains to be elucidated.

Our results of platelet aggregation triggered by agonists at low concentrations are consistent with previously published data showing the neutrality of rivaroxaban and dabigatran with regard to platelet aggregation triggered with standard concentrations [7]. It was previously suggested that dabigatran might enhance PAR-1 density on platelets [8]. However, this effect was observed at a very high dabigatran concentration, 20-fold higher than the therapeutic range.

DOAC inhibit thrombin generation and thus might indirectly delay platelet aggregation. We examined therefore DOAC effect on platelet aggregation secondary to coagulation activation. In this assay, thrombin generated on platelet surface acts as a potent activator of PAR-1 and PAR-4, resulting in platelet aggregation. Dabigatran increased the lag-time and decreased the velocity more significantly than rivaroxaban. This difference probably results from the more potent delay of thrombin generation induced by dabigatran following coagulation activation, as already shown by Rigano et al [9].

Dabigatran and rivaroxaban were already proven not to impact the effect of aspirin on platelet aggregation in treated patients [7]. In return, we demonstrated that aspirin, even at a large excess with regard to therapeutic levels (5–18 μg/ml) did not impact DOAC effect on platelet reactivity following coagulation activation. These results are in accordance with Perzborn et al. study [6] where the combination of aspirin and DOAC had no additional effect on thrombin generation triggered in PRP compared with DOAC alone. It is to mention that besides the inhibition of platelet thromboxane A_2 synthesis, aspirin increases fibrin clot permeability by acetylating lysine residues in fibrinogen enhancing therefore clot lysis and DOAC would unlikely modify this effect.

The net clinical benefit of combining anticoagulants to antiplatelet drugs in preventing atherothrombosis might not be comparable between DOAC. Indeed, we proved that rivaroxaban and dabigatran had different effect on platelet aggregation secondary to coagulation activation. Besides, discrepancy might be due to a mechanism beyond the anticoagulant activity. While being the main targets for anticoagulant drugs, FXa and thrombin also participate in other biological and

Table 1
Effect of DOAC on platelet aggregation induced by agonists at low concentrations. Maximum amplitude (MA %) was normalised to control (i.e. 0.05% DMSO without DOAC). Median of 12 measured values (95% confidence interval) are reported for DOAC plasma concentrations and platelet aggregation parameters.

Agonists		Arachidonic acid (0.5 mg/ml)	Epinephrine (0.5 μM)	ADP (2.5 μM)	TRAP-6 (7.5 μM)	Collagen (0.8 μg/ml)		Ristocetin (1.2 mg/ml)	
Parameters		MA (%)	MA (%)	MA (%)	MA (%)	Lag time (*sec*)	MA (%)	MA (%)	Velocity (%/sec)
Control		100	100	100	100	26 [20–40]	100	100	77 [41–103]
Rivaroxaban (ng/ml)	28 [23–36]	97 [87–106]	97 [80–109]	93 [74–116]	100 [60–125]	26 [19–41]	99 [92–106]	99 [93–111]	78 [63–97]
	128 [119–144]	89 [83–106]	95 [71–123]	94 [83–111]	100 [55–171]	26 [20–40]	102 [84–115]	98 [85–111]	65 [40–100]
	321 [293–361]	101 [80–120]	97 [56–119]	88 [64–117]	105 [92–133]	29 [21–41]	100 [50–128]	104 [91–124]	67 [40–123]
Dabigatran (ng/ml)	39 [34–45]	98 [86–109]	98 [78–116]	98 [88–110]	107 [71–160]	28 [23–42]	95 [66–110]	102 [88–115]	70 [45–93]
	171 [166–193]	98 [85–114]	100 [86–130]	107 [86–130]	108 [91–146]	30 [24–43]	98 [60–118]	102 [92–119]	74 [46–104]
	353 [349–382]	97 [87–106]	92 [26–156]	95 [62–122]	97 [79–111]	32 [22–43]	90 [31–118]	99 [83–112]	72 [42–104]

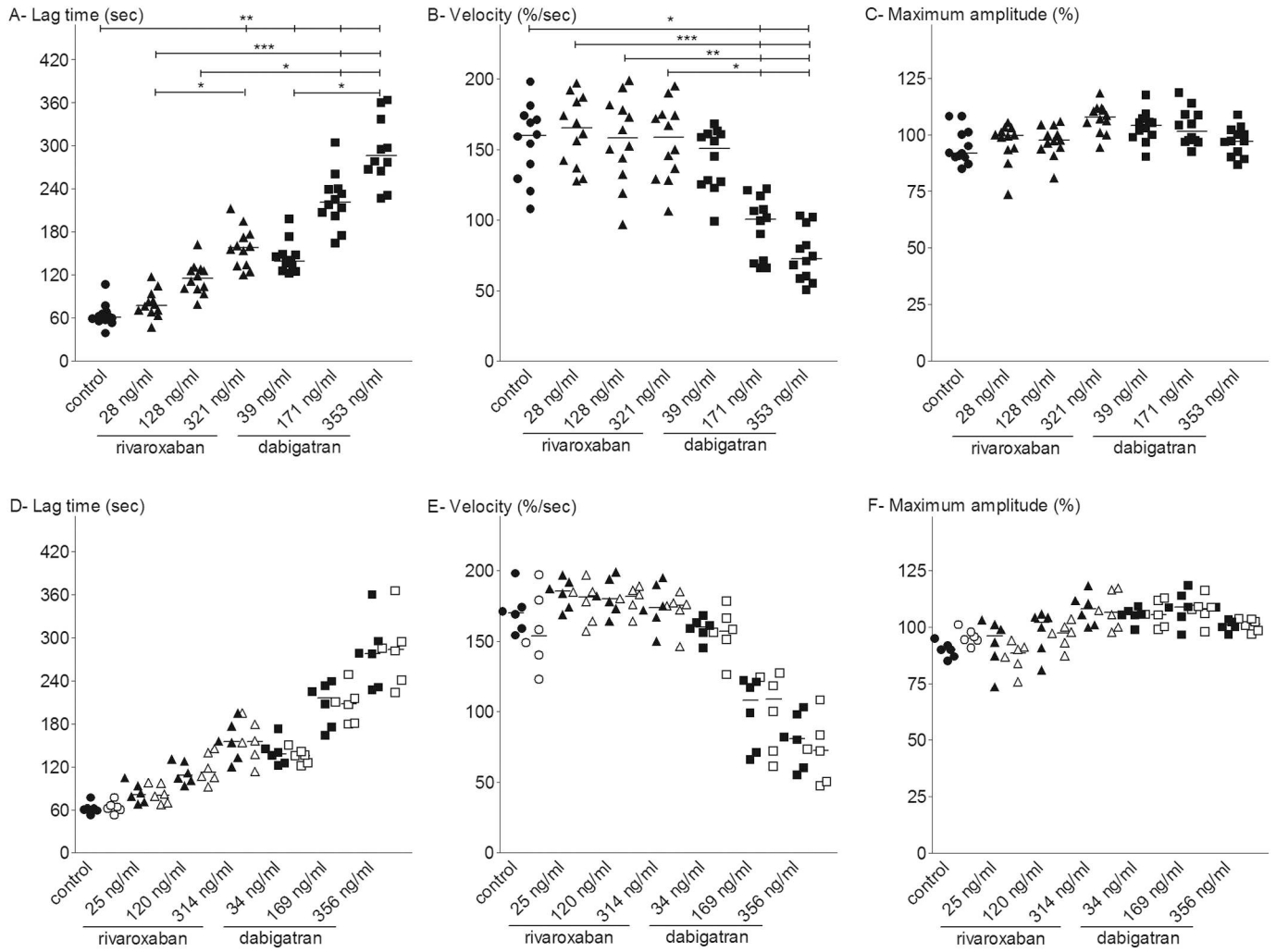

Fig. 1. Effect of DOAC on platelet aggregation *sec*ondary to coagulation activation in the absence or presence of aspirin. Coagulation was triggered in platelet-rich plasma of 12 healthy volunteers (A, B, C) with 0.5 pM tissue factor and $CaCl_2$ in the presence of GPRP to prevent fibrin polymerization. Three increasing concentrations of rivaroxaban (triangles) or dabigatran (squares) were tested. They were measured by specific assays in plasma. Median values are indicated in the X axis. Aspirin (open symbols) or vehicle (closed symbols) was thereafter added to PRP from 6 out of 12 healthy volunteers in the presence of DOAC (D, E, F). Three parameters were analysed: lag time (sec), velocity (%/sec) and maximum amplitude of platelet aggregation (%) normalised to control. Lines represent median values. A concentration dependent increase of lag time was observed in the presence of DOAC (A). Dabigatran delayed platelet aggregation more significantly than rivaroxaban (B). Velocity was only decreased with high dabigatran concentrations while maximum amplitude was not affected in the presence of DOAC (C). Aspirin has no impact on rivaroxaban or dabigatran effect on any studied parameter (D, E, F).*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

pathophysiological processes including inflammation, angiogenesis and atherosclerotic plaque development via mechanisms independent of thrombus formation promotion [10]. FXa and thrombin pleiotropic effects might likely be altered in the presence of DOAC with different impacts on atherothrombotic risk. Indeed FXa inhibition by rivaroxaban results in a down-regulation of thrombin generation altering therefore the pleiotropic effects of the latter, while thrombin inhibition by dabigatran will not impact FXa effects outside the coagulation system.

Our study has some limitations. DOAC were added in vitro to whole blood samples. Although unlikely, any effect on megakaryocytes or platelet production cannot be excluded. Apart from that, the number of volunteers tested may have limited the possibility to detect any effect of DOAC on platelet reactivity; to overcome this, we have triggered platelet aggregation by standard agonists at low doses and have tested 3 increasing concentrations per DOAC. Furthermore, testing in vitro spiked samples from healthy volunteers is a limitation of our study; however, it allows avoiding any potential risk factor for platelet activation observed in cardiovascular patients. This clinical situation might be responsible of the altered ex vivo platelet function in DOAC treated patients reported in a limited number of previously published studies.

In conclusion, the positive benefit-risk balance in atherothrombosis prevention obtained with the combination of DOAC and aspirin in contemporary clinical trials does not appear to stem from a direct antiplatelet effect of DOAC as shown in our in vitro study. This combination would have an additive inhibition effect on platelet activation: directly by inhibiting thromboxane A_2 synthesis with aspirin and indirectly by limiting platelet aggregation secondary to coagulation activation as a consequence of a direct inhibition of thrombin with dabigatran or of its generation with xabans. Additional therapeutic potential of DOAC might also result from the alteration of the pleiotropic functions of the targeted serine proteases, which remains to be elucidated.

Declaration of competing interest

P. Gaussem has received honoraria for participating in expert meetings on rivaroxaban (Bayer Healthcare AG). The other authors declare no conflict of interest.

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