Assessing the influence of diurnal variations and selective Xa inhibition on whole blood aggregometry

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Abstract
A biological rhythm in platelet function is well known. Multiple electrode aggregometry (MEA) is a widely used assay to measure platelet aggregability. Rivaroxaban is a new oral anticoagulant frequently used in an increasing number of indications. In this randomized, crossover trial we investigated whether a biological rhythm exists in MEA measurements and potential effects of rivaroxaban on platelet aggregation. Sixteen healthy volunteers were included in the study and blood samples were obtained at 08:00, 12:00, 16:00 and 20:00 h. Each subject was tested without rivaroxaban intake first and randomly assigned to 3 days of rivaroxaban intake at 08:00 or 3 days of rivaroxaban intake at 20:00 h and vice versa. In MEA measurements, a significant increase in platelet aggregation after addition of ristocetin at 12:00 h compared to other investigated time-points (122±8 AU at 12:00 h vs. 109±9 AU at 08:00 h, 114±10 AU at 16:00 h and 103±8 AU at 20:00 h, p = 0.027) could be detected. There was no biological rhythm detectable using other agonists (ADP, arachidonic acid, thrombin-receptor activating peptide-6). After rivaroxaban intake at 08:00 h an increased ristocetin-induced platelet aggregation was measured in the next morning (126±4 AU (rivaroxaban at 08:00 h) vs. 109±9 AU (no rivaroxaban), 111±6 AU (rivaroxaban at 20:00 h; p = 0.002). No other effects of rivaroxaban on platelet function were found. We detected a biological rhythm in ristocetin-induced platelet aggregation with a peak at 12:00 h (noon). No influence of selective Xa inhibition on platelet aggregation was detected.

Key Words: Arachidonic acid, ADP, circadian rhythm, platelet aggregation, ristocetin, rivaroxaban, TRAP peptide, von Willebrand Factor, factor Xa

Introduction
Biological rhythms in coagulation, defined by varying levels and activities of coagulation or fibrinolysis factors and platelet function over a 24-h time-period, is an intensively studied topic, which is not just of academic interest, but comprises important implications for clinical routine, i.e. it might affect drug dosing schedules. For instance, plasma concentrations of hemostatic factors, among others prothrombin fragment F1 + 2, plasminogen activator inhibitor 1, factor VIIa, factor VIII activity, von Willebrand factor (vWFP) or fibrinogen, are all significantly higher during the morning hours compared to the evening [1,2]. There are also rhythmic changes in platelet function, with maximum platelet activation in the morning period [3]. Taken together, the rhythmic properties of hemostatic factors result in a prothrombotic state in the morning. This is also reflected in the greater incidence of thromboembolic events during the morning hours [4,5].

Moreover, biological rhythms in pharmacokinetics and pharmacodynamics of various anticoagulant drugs were intensively investigated [2]. For instance, continuous heparin infusion has varying anticoagulant effects during 24 h with a striking decreasing effect in the morning hours [6].

Multiple Electrode Aggregometry (Multiplate, MEA) is a semi-automated, frequently used method to analyze platelet aggregation after the addition of various agonists. ADP or arachidonic acid [7] are...
agonists added to measure the pharmacodynamic effect of antiplatelet drugs, such as aspirin or P2Y12-inhibitors [8]. Ristocetin (RISTO) addition is used for the analysis of the vWF- and glycoprotein Ib (GpIb)-dependent platelet aggregation. Thrombin receptor activating peptide-6 (TRAP) is used to measure platelet aggregation via the thrombin receptor PAR-1.

Rivaroxaban is a recently marketed, oral, selective, direct factor X-inhibitor usually dosed once per day, that was non-inferior to warfarin in the prevention of stroke or systemic embolism in patients with non-valvular atrial fibrillation [9]. The effects of rivaroxaban on platelet function have not been analyzed so far in vivo. However, rivaroxaban did not exert any effects on platelet function in vitro [10]. Thus, in vivo possible differences between rivaroxaban intake in the morning or in the evening remain to be investigated.

The aim of this study was to investigate a possible biological rhythm in platelet aggregation assessed by whole blood aggregometry. Diurnal variations of VWF-antigen and activity were recently demonstrated by Timm et al. [11] and VWF-antigen will also be assessed in this project. In addition we aimed to analyze effects of rivaroxaban on platelet function, as well as the impact of morning or evening administration.

We hypothesized that: (i) A biological rhythm in platelet aggregation may be detectable by whole blood aggregometry, and (ii) that selective anti-Xa inhibition does not exert any effects on platelet aggregation in vivo.

Methods

This randomized, controlled, analyst-blinded trial was performed at the Department of Clinical Pharmacology at the Medical University of Vienna between March and May 2014. The study protocol was approved by the independent ethics committee of the Medical University of Vienna. The study was conducted in accordance with the Good Clinical Practice guidelines and the Declaration of Helsinki.

Subjects

Sixteen healthy male and female volunteers aged between 18 and 65 years were included in the trial. All participants signed an informed consent form before any trial related activity was performed. All female subjects of childbearing potential had a negative urine pregnancy test before inclusion. Inclusion criteria were normal blood pressure (<140/90 mmHg after 5 min in a supine position); absence of relevant diseases and normal laboratory values; no jet lag, shift work or sleep disorders interfering with a normal chronobiological routine within 2 weeks before the first study day; no history of anticoagulant treatment or elevated bleeding risk within 6 months before the first study day. Among exclusion criteria were renal or hepatic impairment, pregnancy, blood/plasma donation within 4 weeks, alcohol or drug abuse, non-compliance with the study and lifestyle requirements, history or presence of relevant diseases and intake of any drugs one week prior to the first trial day.

Study design

Sixteen healthy volunteers were block-randomized to two different study periods. The first period started with a baseline assessment. Subjects were confined to the study ward from 08:00–20:00 h after an overnight fast and fresh venipunctures were performed every 4 h (±15 min), corresponding to 08:00, 12:00, 16:00 and 20:00 h. MEA measurements were performed at these time-points and blood samples were frozen for further analysis as explained in detail below. All subjects received standardized, fat reduced meals during the trial day after the venepunctures at 08:00 h (±30 min), at 12:00 h (±30 min) and at 16:00 h (±30 min).

If subjects were assigned to the first study group, they received the first dose of 10 mg rivaroxaban after the last venipuncture at 20:00 h (±15 min), and continued to take rivaroxaban at 20:00 h (±15 min) for another two days. After three evening doses, on day four subjects MEA analysis was performed again at 08:00 h (12 h after the last dose).

After a minimum three-day wash-out period, subjects returned to the study ward at 07:30 h and started a three-day course of 10 mg rivaroxaban at 08:00 h. MEA analysis was again performed at 08:00 h, approximately 24 h after the last dose.

The second study group started with the baseline assessment, followed by three rivaroxaban doses in the morning. After a three-day washout period all subjects received 10 mg rivaroxaban at 20:00 h for three consecutive days and MEA measurements were performed thereafter (also see the flow-chart in Figure 1).

Platelet function testing

Venous blood samples were drawn at predefined endpoints in commercially available hirudin anticoagulated blood sampling tubes (4 mL, >15 μg/mL, Vacuette). For our testing system no difference between arterial and venous blood samples was detected [7].

Platelet function was assessed by the Multiple Electrode Aggregometry (MEA, Multitherplate Roche) as previously described [12–14]. In short, whole blood was diluted with 0.9% NaCl in a test cuvette and automatically mixed for 3 min at 37°C. We
analyzed platelet function using the following activators: adenosine-diphosphat (ADP, 6.4 μM), arachidonic acid (AA, 0.5 mM), ristocetin (RISTO, 0.77 mg/mL) and thrombin receptor activating peptide-6 (TRAP, 32 μM). The system measures the increase in electrical impedance due to adhesion or aggregation of platelets in arbitrary units (AU) for a period of 6 min after addition of the activator. Results correspond to the area under curve (AU*min) and are presented AU. All blood samples were analyzed after 30 min but within 4 h as suggested by the manufacturer. The inter-individual coefficient of variation in a previous study including 20 healthy volunteers was 29.5%, 20.2%, 30.5% and 16.3% for ADP, AA, RISTO and TRAP test, respectively [15].

vWF measurement

Blood samplings were performed at predefined time-points during the baseline assessment study day. Citrate-anticoagulated sampling tubes were used (3.8% Citrate, 3.5 mL, Vacuette). After blood collection, tubes were centrifuged for 10 min at 2000 g and plasma was frozen. Levels of vWF were measured using marketed enzyme-immuno assays (vWF Ag Elisa, Dade Behring). Results will be presented in %. All assessments were performed according to the manufacturer’s instructions.

Statistical analysis

All data are expressed as means ± standard error of the mean (SEM) unless otherwise stated. We performed Friedman-ANOVA for overall differences between time-points or groups, whereas pairwise comparisons were conducted by a t-test for related samples. A two-tailed significance level of p < 0.05 was considered statistically significant. All statistical calculations were performed using commercially available statistical software (IBM SPSS Statistics Version 22). All tests were performed on original data.

Results

Baseline

Sixteen healthy volunteers were included in the trial and all participating subjects completed the study visits as planned. Baseline data are presented in Table I.

Platelet function

Biological rhythm. MEA measurements were performed at four pre-defined time-points as explained above. No significant difference in platelet function was detectable using the agonists ADP (p = 0.336), AA (p = 0.913) or TRAP (p = 0.602) between the time-points. A statistical significant difference was found in platelet function using RISTO (overall p = 0.027). Results are presented in Figure 2. Results of the ristocetin-induced platelet aggregation were 109 ± 9 AU at 08:00 h; 122 ± 8 AU at 12:00 h; 114 ± 10 AU at 16:00 h; and at 20:00 h 103 ± 8 AU. To correct for differences in baseline values all tests were performed as relative changes from the baseline.

There was a significant increase in platelet aggregation between 08:00 and 12:00 h (p = 0.018) and a

Table I. Baseline data.

| Gender m (f) | 13 [3] |
|-------------|--------|
| Age (years) | 27 ± 2, |
| Height (cm) | 178 ± 2 |
| Weight (kg) | 75 ± 4  |
| BMI (kg/m²) | 23.4 ± 0.7 |
| Hemoglobin (g/L) [f: 120–160, m: 135–185][21] | 148 ± 28 |
| Platelet count (10⁹/L) [150–350] | 251 ± 9 |
| White blood count (10⁹/L) [4–10] | 5.7 ± 0.3 |
| Prothrombin time (s) [26.7–37.9] | 37.3 ± 1.1 |
| Activated partial thromboplastin time (s) [<49 s] | 36.0 ± 0.8 |
| Fibrinogen (g/L) [1.8–3.9] | 2.65 ± 0.15 |

Data are presented as means ± standard error of the mean, reference values are presented in the brackets. BMI, Body mass index.
significant decrease between 12:00 and 20:00 h \((p = 0.013)\) in the RISTO test. No significant differences were found between any of the other tests or time-points. The intraindividual coefficient of variation was 12%, 11%, 18% and 14% for ADP, AA, RISTO and TRAP, respectively.

**Influence of selective Xa inhibition on platelet function.** A significantly increased platelet aggregation after addition of ristocetin was measured when 10 mg rivaroxaban were taken at 08:00 h compared to intake at 20:00 h \((p = 0.016)\) or the baseline assessment \((p = 0.024)\). At 12:00 h no difference was detectable between morning intake, evening intake or no intake. As presented in Table II, selective Xa inhibition did not affect platelet aggregation induced by ADP, AA, RISTO and TRAP.

**vWF-Ag levels.** Overall a significant difference between all time-points could be found \((p = 0.002)\).

Comparing the time-points with each other showed significantly higher levels at 08:00 and 12:00 h compared to 16:00 h \((p = 0.001)\, for\ both\ tests, Table III). We measured a borderline significant trend towards higher levels at 20:00 compared to 16:00 h \((p = 0.06)\) (Figure 3). No significant difference was found between the other time-points.

**Discussion**

This study presents several new findings: (i) Platelet aggregation in response to ristocetin addition displays a biological rhythm, with a peak at 12:00 h (noon). No such rhythm can be found regarding the other agonists ADP, AA or TRAP; and (ii) selective Xa inhibition by rivaroxaban intake does not interfere with platelet function testing.

The accumulation of thromboembolic events during the morning hours led to a large number of studies investigating circadian rhythms in coagulation and hemostasis. The results of these projects suggest a prothrombotic state in the morning. This may also hold true for platelet function [16]. The implications of these findings are of clinical

**Table II. Influence of 10 mg rivaroxaban on platelet function during morning blood sampling at 08:00 h.**

|                   | No Rivaroxaban | Rivaroxaban 08:00 h | Rivaroxaban 20:00 h | \(p\)-value |
|-------------------|----------------|---------------------|---------------------|-------------|
| ADP               | 65 ± 3         | 69 ± 3              | 65 ± 4              | 0.12        |
| AA                | 84 ± 4         | 88 ± 4              | 88 ± 4              | 0.647       |
| RISTO             | 109 ± 9        | 126 ± 4*            | 111 ± 6             | 0.015*      |
| TRAP              | 94 ± 5         | 96 ± 2              | 94 ± 5              | 0.47        |

ADP, ADP-induced platelet aggregation; AA, arachidonic acid-induced aggregation; RISTO, ristocetin-induced aggregation; TRAP, thrombin receptor activating protein-6-induced aggregation. Results of Multiplate Electrode Aggregometry in arbitrary units \((\text{AU}^* \text{min})\), data are presented as means ± standard error of the mean.
importance. For example, it was demonstrated that the timing of blood sampling matters, to identify high on treatment platelet reactivity in patients receiving dual antiplatelet therapy. The amount of patients with poor pharmacodynamic response to antiplatelet therapy at 10:00 h was significantly higher compared to other time-points [17]. Moreover, closure times measured by the platelet function analyzer investigating the platelet function under high shear rates, is shortest in the morning and lengthens in the afternoon, indicating higher vWF levels in the morning [18]. MEA is widely used to test the pharmacodynamic response to antiplatelet drugs, particularly in the use of ADP-receptor inhibitors. To our knowledge, this is the first study to investigate a possible biological rhythm in platelet aggregation measured by the MEA system. Our results suggest that no such biological rhythm exists in the ADP, the AA or the TRAP test. However, we detected a peak in platelet aggregation after addition of ristocetin at 12:00 h. This modest but significant increase in platelet aggregation may represent a higher activity of the vWF at that specific time of the day (Figure 2). However, the increase in platelet function was overall modest and the clinical importance is at least arguable. If a laboratory uses the MEA-RISTO test for diagnostic workup of the von-Willebrand-disease changes due to this biological rhythm may affect the diagnostic performance in case of borderline values. The baseline values in general are in line with other studies. A recent study including 72 healthy volunteers measured mean baseline values of the RISTO test of 110 (± 33, standard deviation) [19]. This is in good agreement with our results and support their external validity.

The intra-individual coefficient of variation was 11–18%, the inter-individual coefficient of variation was 20–23% for different agonists in our trial. We had 80% power to detect a single standard deviation difference from baseline in measured outcome parameters, i.e. an 11–18% change in aggregation in response to different agonists. This is in good agreement with the reported inter-individual coefficient of variation of 20–30% of a previous trial of our working group [15] and intra-individual coefficient of variation of about 10% in other trials [20,21].

The circadian rhythm of the vWF-antigen and activity was previously examined by Timm et al. [11]. In their study, levels of vWF antigen were also highest during the morning hours, with a drop at around 15:00 h and higher levels in the evening. During the night vWF antigen levels decreased again to lower levels. This is in line with our results that lowest levels of vWF antigen were found at 16:00 h with almost equally high values at 08:00, 12:00, and 20:00 h (Figure 3).

Rivaroxaban is a direct inhibitor of factor Xa of the coagulation cascade and does not primarily inhibit platelets. Thus, we did not expect large changes in platelet aggregation after intake of rivaroxaban. However, rivaroxaban is also approved for the use in secondary prevention of acute coronary syndromes and in this setting usually is combined with dual antiplatelet therapy. Whole blood aggregometry is widely used for therapy monitoring of ADP-receptor inhibitors clopidogrel, prasugrel and ticagrelor. To date, only one in vitro study exists investigating the effects of selective Xa-inhibition on various coagulation and platelet function tests [10]. We now extend these findings with in vivo data. Selective Xa-inhibition does not interfere with the results of the MEA system. Thus, therapy monitoring of ADP-receptor inhibitors may be performed. We consider our finding that rivaroxaban intake in the morning results in a higher ristocetin-induced platelet aggregation to be most probably due to chance. Again, the magnitude of this effect is moderate and clinical implications are questionable.

Limitations
The study population of 16 healthy volunteers is relatively limited and we cannot exclude that some of our results may be due to statistical chance. This especially holds true for the RISTO test as the range of results was rather large (67–173 at the baseline). Accordingly, the range of baseline vWF-Ag levels was large (53–155%). Our study setting included only four blood sampling time points between 08:00 and 20:00 h and we did not investigate possible diurnal variations during the night. Thus, we cannot exclude circadian variations in platelet aggregation during the night.

Conclusion
Reliable measurements of MEA are possible in the presence of rivaroxaban, because, except for a small
effect on ristocetin-stimulated aggregation, it does not interfere with the results of the testing system using other agonists.

Acknowledgements

The study was supported by the Bürgermeisterfonds of the city Vienna. C.S. and M.S. were supported by the FWF (Fonds zur Förderung der wissenschaftlichen Forschung), grant number 14005. The authors would like to thank the laboratory technicians Christa Drucker and Karin Petroczi for technical assistance and study nurse Edith Lackner for her contribution to the conduct of the study.

Declaration of interest: Bernd Jilma has been acting as the principal investigator of several studies sponsored by Bayer AG. The authors report no other conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Supplementary material available online

Supplementary Appendix Figure 1 to be found online at http://informahealthcare.com/doi/abs/10.3109/00365513.2015.1057896.