

CLINICAL STUDY

The effect of warfarin administration on platelet aggregation

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ABSTRACT

BACKGROUND: The aim of this study was to assess the effect of anticoagulation treatment on platelet aggregation.

METHODS: The study group consisted of 24 patients on long-term warfarin therapy without any antiaggregation therapy. Platelet aggregation was measured using VerifyNow with arachidonic acid (AA) as an inducer in 23 patients and with light transmission aggregometry (LTA) in 19 patients using four different agonists. All patients had their international normalized ratio (INR) checked regularly.

RESULTS: The mean INR value was 2.07 (SD 0.6). The average aggregation measured by VerifyNow was found to be 637.5 (SD 36.6) aspirin reaction units. The values of average aggregability in LTA were 73.3 % (SD 4.5 %), 73.2 % (SD 6 %) and 72.1 % (SD 4.8 %) in case of aggregation induced by AA, ADP, and collagen, respectively. Epinephrine-induced aggregability was 65.3 % (SD 14.7 %). Regression analysis between INR and values of collagen- or epinephrine-induced aggregability ($r = 0.654$ and 0.575) was found statistically significant ($p = 0.004$ and 0.016); every increase in INR by 0,1 brings about an increase in collagen- and epinephrine-induced aggregation values by 1.5 and 4, respectively.

CONCLUSION: Administration of warfarin does not produce a significant reduction in platelet aggregation. On the contrary, prolonged INR evokes a mild increase in aggregation induced by collagen or epinephrine (Tab. 2, Fig. 3, Ref. 32). Text in PDF www.elis.sk

KEY WORDS: platelet aggregation, anticoagulation, warfarin, platelet function tests, chronic ischemic heart disease.

Introduction

Oral anticoagulation with warfarin is recommended in the treatment of venous thromboembolism, as well as in order to reduce the risk of stroke in patients with atrial fibrillation, atrial flutter, or valvular heart disease (1).

Under normal circumstances, proteins including the coagulation factors II, VII, IX, and X require carboxylation by vitamin K to obtain proper biological activity. Warfarin, a coumarin derivative, induces hepatic production of partially decarboxylated proteins with reduced coagulation activity by inhibiting the vitamin K conversion cycle (2).

In thrombogenesis two participating processes, namely platelet aggregation and activation of coagulation are closely linked. Despite that, in medical practice, both these particular mechanisms are usually viewed separately and consequently, they are also measured and treated as two isolated categories.

The dichotomy is relevant in patients on anticoagulation or antiaggregation drugs. Considering the tight binding of aggregating thrombocytes to each other via fibrin bridges, we could speculate that any intervention in the coagulation cascade would weaken the mutual adhesion of thrombocytes and thus encumber the efficacy of aggregation.

Haematologic literature contains several observations on the dependency of aggregation on coagulation-suppressing drugs. Such studies have been appearing since the seventies, particularly with heparin, and they reported an induction of increased aggregation along with heparin-induced thrombocytopenia (3, 4, 5, 6), or inhibition of platelet accumulation (7), especially with high doses of heparin (6). More recent studies postulated that warfarin has a stimulating (8) or no effect on platelet aggregation (9, 10). However, studies with warfarin are quite rare. The new anticoagulant drugs (NOAC) have been investigated more frequently. Some reveal a reduced platelet aggregability along with suppressed coagulation using dabigatran (11, 12, 13) as well as xabans (14). However, namely with dabigatran, the reports on its effect on aggregability differ. Some authors show an increase (15) while others report no effect (16, 17). Beside the human *in vitro* and *ex vivo* studies of

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Tab. 1. Baseline characteristics of our patients (n¹ = 24).

Mean age (range)	79 (58–92)
Male/female	14/10
Arterial hypertension	11 (46 %)
Pacemaker or implantable cardioverter defibrillator	24 (100 %)
Arrhythmia	19 (79 %)
Valvular disease	6 (25 %)
Coronary artery disease	9 (38 %)
Peripheral arterial disease	3 (13 %)
Diabetes mellitus	8 (33 %)
Hyperlipidaemia	16 (67 %)
Number of thrombocytes	187.5 (SD ² 60.6) × 10 ⁹ /L

¹n = number of patients ²SD = standard deviation

aggregation influenced by heparin, there are some experimental animal studies carried out *in vivo* (6).

The comparison of the above-mentioned studies is difficult, since they were carried out with different patient groups, inducers, or aggregometry methods. To obtain our own results of the platelets' readiness to aggregate when influenced with warfarin, we have carried out a study on patients with chronic ischemic heart disease. The purpose of the study was to assess the effect of anticoagulation treatment with warfarin on platelet aggregation in patients with pacemakers or implantable cardioverter defibrillators.

Material and methods

This study was approved by the ethical committee of the University Hospital Královské Vinohrady, Prague, Czech Republic, and was performed according to the principles of the Declaration of Helsinki. Written informed consent was obtained from all participants.

The platelet aggregation was measured by two different methods, namely by means of bedside optical aggregometry with the VerifyNow system (Accumetrics Inc., San Diego, Calif.) in full blood with arachidonic acid (AA) as an inducer, and by using light transmission aggregometry (LTA) in platelet rich plasma, while using four different agonists – AA, collagen, epinephrine, and adenosine diphosphate (ADP).

The results were expressed in a) aspirin reaction units (ARU) in VerifyNow, where values lower than 550 ARU are considered to be indicative of reduced aggregability (18), and b) LTA as percentage of aggregation (in fact change in light transmission) with < 55 % indicating suppressed aggregation of platelets (19).

All patients were on long-term warfarin therapy with regularly checked INR twice per month. The whole study group consisted of 24 (14 males) patients with pacemakers or implantable cardioverter defibrillators, while being on long-term warfarin therapy and no antiaggregation therapy. Platelet aggregation was measured using VerifyNow in 23 patients and light transmission aggregometry (LTA) was carried out in 19 patients.

The baseline characteristics of the study population are presented in Table 1. No subject had suffered from any recent ischemic event. Users of any other anticoagulant drugs and persons suffering from acute diseases were excluded. The compliance with warfarin intake was ensured by INR levels and face-to-face interviews.

Blood sampling and processing

Blood for the VerifyNow system was drawn into 2-mL tubes (Greiner Bio-One Vacuette®) with 3.2 % sodium citrate. The samples were collected atraumatically under standardised conditions using direct venous puncture, while taking care to avoid contamination by tissue factors (free extremity, no retraction). Using a 21-gauge or larger needle, we first collected a volume of at least 2 mL of blood, which was discarded. We then collected a further volume of 2 mL of blood in a sample tube. The sample tube was gently inverted at least five times to ensure complete mixing of the contents. Samples with evidence of clotting were not used. After the collection, the samples were maintained at room temperature and then incubated for 30 minutes. All assays were performed according to the manual, i.e., within 2 to 3 h of sampling (18).

Blood for LTA was drawn into 5-mL tubes containing one part of 3.8 g/ dl sodium citrate for nine parts of venous blood. The samples were taken in the same manner but we collected a volume of 5 mL of blood in three sample tubes. Each sample tube was gently inverted at least five times to ensure complete mixing of the contents. After collection, the samples were maintained at room temperature and then incubated for 30 minutes. All assays were performed according to the manual, i.e., within 1 h of sampling (19).

All platelet aggregation analyses were performed in the morning hours to exclude diurnal changes. We used vacuette tubes containing ethylenediaminetetraacetic acid (EDTA) to measure haemoglobin and platelet counts. Blood for biochemical analyses (especially lipid profile, renal and liver functions, and markers of inflammation) was collected in vacuette tubes without additives, and blood for coagulation analysis was collected in vacuette tubes containing 3.2 % sodium citrate.

Platelet aggregation measurement

The point-of-care optical aggregometry VerifyNow system (Accumetrics Inc., San Diego, Calif.) was used with arachidonic acid (AA) as an inducer. The results are expressed in aspirin reaction units (ARU). Prior to the insertion into the cartridge that was pre-mounted to the instrument, the blood samples were mixed simply. The aggregation in response to the agonist was monitored by light transmission through two duplicate reaction chambers in each cartridge. Cartridges, routinely used for responses to acetylsalicylic acid (ASA), contained AA as a platelet activator and fibrinogen-coated beads. The instrument provides immediate information without any need for transport or delays in special laboratories (20). The results are reported in ARU, with a maximum value of 700 and a minimum value of 350. Values higher than 550 ARU were indicative of 'no effect' or high on-treatment reactivity, and values below 550 were interpreted as 'reduced aggregability' (18).

Light transmission aggregometry (APACT LPC Software, Version 1.21c, Fa. LABiTec) was performed with AA, collagen, ADP, and epinephrine as inducers. The results are expressed in percentages. The 100 % line was set equal to platelet-poor plasma (PPP) and the 0 % baseline was established equal to platelet-rich plasma (PRP) prior to the addition of any agonist. PRP was prepared by centrifugation for 15 minutes at 163 g. The aim was to

Tab. 2. Pearson’s correlation coefficient between INR and aggregation using 4 inducers in LTA and using VerifyNow (VN).

	INR		Age	
	R	P-value	R	P-value
ADP	0.450	0.070	0.201	0.440
Collagen	0.654	0.004 ²	-0.059	0.821
Epinephrine	0.575	0.016 ¹	-0.097	0.710
AA	0.425	0.089	0.011	0.966
VN	0.110	0.636	-0.304	0.181

¹significant at the 0.05 level, ²significant at the 0.01 level

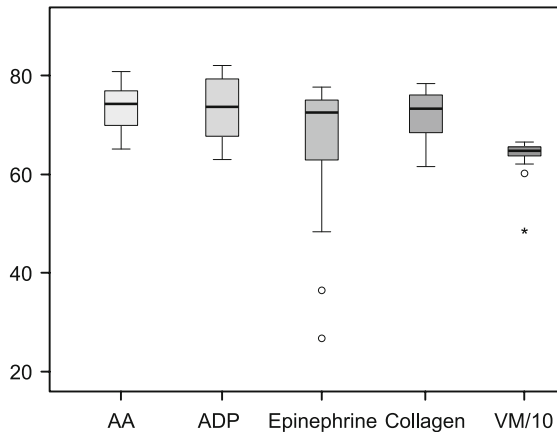


Fig. 1. Level of aggregation in patients on long-term warfarin therapy. Results of LTA using four different agonists (AA, ADP, epinephrine and collagen) are shown as percentage of light transmission. Those of VerifyNow (VerifyNow Measurements in ARUs/10 = VM/10) are in ARU units.

concentrate the platelets to values recommended in the literature, i.e., between 200 and 400 x 10⁹/l (19, 21). PPP was prepared by further centrifugation for 15 minutes at 2500 g. For very high counts, the specimen was adjusted to optimum by PPP. PRP was stirred in 200 µL minicuvettes at 37.4 °C and each cuvette was placed between the light source and photocell. The addition of a platelet agonist to PRP provokes the platelet activation and brings about a change in their shape from discoid to spiny spheres, while reducing the absorption of light by platelets. After one of the four agonists mentioned above is added, the platelets aggregate and thus absorb less light, which increases the transmission detected by the photocell (19, 20). Four different inducers, namely AA, collagen, epinephrine, and ADP (in final concentrations of 0.5 mg/mL, 10 µg/mL, 10 µmol/L, and 20 µmol/L, respectively) were individually added. The shape of the curves and percentage of aggregation were recorded (19, 22, 23, 24, 25). In LTA, the limit of sufficient reduction of aggregation after treatment was set to the value of < 55 % according to literature, where such values are broadly reported for all agonists. These limits are in agreement with the limits of our laboratory.

Statistical analysis

The first statistical step was the comparison of the whole averaged cohorts in particular aggregometry methods. Next, we per-

formed regression models to test the linear relationship between aggregation using four different inducers as dependent variables and INR as an independent variable. Prior to statistical testing, the Dean-Dixon test was used to detect outliers in a univariate data set. Furthermore, the age of patients was included as a second regressor in the models. The Pearson correlation coefficient *r* and coefficient of determination *R*² were used to quantify the degree of association. ANOVA and t-test were used to test the significance of linear regression model. The statistical significance was set to 5 %. The data were processed using IBM SPSS Statistics for Windows, version 24 (IBM Corp., Armonk, N.Y., USA).

Results

All of our patients had effective INR levels. The mean INR value was 2.07 (SD 0.6). The mean platelet aggregation in patients on long-term warfarin therapy was 637.5 (SD 36.6) ARU using VerifyNow. The mean aggregability values in LTA with AA, ADP and collagen were 73.3 % (SD 4.5 %), 73.2 % (SD 6) and 72.1 % (SD 4.8), respectively. Epinephrine-induced aggregability was 65.3 % (SD 14.7). Thus, these values did not reach the limits of our laboratory in terms of a significantly reduced standard aggregation (Fig. 1).

To decipher the relation between anticoagulation and aggregability in greater detail, we have performed regression analysis for individual persons. While in VerifyNow and on arachidonic acid or ADP as inducers, no significant correlation was found, the aggregation induced by collagen, and even more that induced by epinephrine appeared to be significantly dependent on warfarin (Tab. 2).

The model explains 42.7 % and 33.1 % of data variability by the use of collagen and epinephrine inducers, respectively. The data indicate that every increase in INR by 0.1 results in a statistically significant increase in the collagen-induced aggregation by 1.5 (p = 0.004). Similarly, an INR increase by 0.1 is accompanied by an increase in epinephrine-induced aggregability by 4.0 (p = 0.016) (Figs 2 and 3).

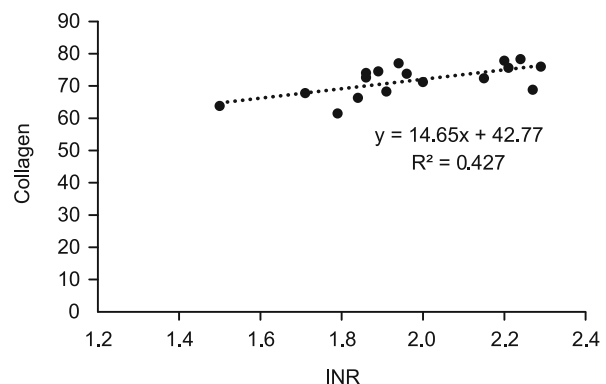


Fig. 2. Linear regression relationship between collagen and INR. Results of LTA using collagen as agonist are shown as percentage of light transmission. INR values are plotted on the x axis.

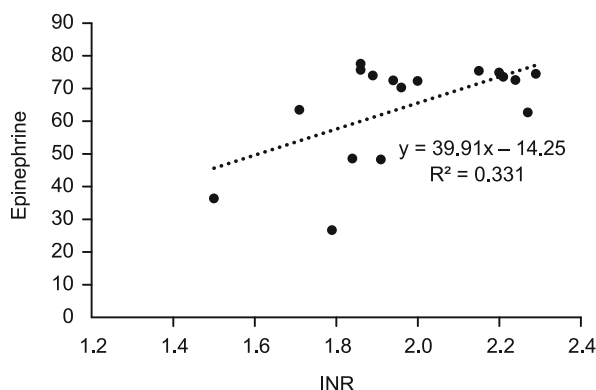


Fig. 3. Linear regression relationship between epinephrine and INR. Results of LTA using epinephrine as agonist are shown as percentage of light transmission. INR values are plotted on the x axis.

Discussion

Already eighty years have elapsed from the time when the scientists started questioning the relation between the two main components of blood clotting, namely coagulation and platelet aggregation (26). Yet, during that time, the interconnection of these mutually dependent and intertwined processes has not been disentangled. In medical practice, coagulation is usually measured separately and more reliably as an aggregation of thrombocytes.

It is well known from meta-analytic studies that adjusted doses of warfarin and antiplatelet agents have reduced the risk of stroke in patients with atrial fibrillation by approximately 60 % and approximately 20 %, respectively (27). Many patients with atrial fibrillation and ischemic heart disease who are treated with anticoagulation agents also need antiaggregation treatment due to other diseases such as atherothrombosis as well as due to the risks of ischemic heart disease or stroke. We usually do not add antiaggregation treatment in these patients due to the potential risks of serious bleeding complications. In this study, we were interested if the platelet aggregation in these anticoagulated patients would be influenced as well and if we would be able to measure this effect.

Our previous experience has taught us that aggregometry is not a simple and one-way process (28, 29). The methods do not measure any spontaneous run of changes, but depend on the induction of aggregation by its particular stimulants. We were interested in finding whether the warfarin-induced haemocoagulation would entail a simultaneous measurable derangement of aggregation provoked by several particular inductors. For this reason, in persons on warfarin, we have analysed aggregometry with LTA using AA, ADP, collagen and epinephrine and by point-of-care test VerifyNow with AA as agonist.

The results of the whole group when presented as average were similar to the aggregation values in healthy individuals without any antiaggregation or anticoagulation treatment (28, 29). Administration of warfarin does not produce a significant reduction in platelet aggregation. Similar results were described previously (30, 31).

After we had deployed the regression model of intraindividual comparison, some correlation became apparent. In case of aggrega-

tion induction by collagen and mainly by epinephrine, a clear effect of prolonged INR on the increased aggregability could be registered. It seems that because these pathways serve as a rescue mechanism in case of stress or insult to the vessel wall, not only the aggregation is unaffected, it increases even despite warfarin therapy. In case of aggregation induction by ADP and arachidonic acid, no substantial dependence was found; these agonists are in play for different drugs' pathways, and in warfarin patients, we were not able to find significant correlations.

We assume that platelet aggregation is spurred by manifold pathways. The use of different agonists in experiment provides disparate results. This may have caused inconsistent findings in our own study, as well as in a number of similar experiments in literature. It is possible that using the thrombin receptor-activating peptide (TRAP) as the most potent agonist of platelet activation via protease-activated receptors 1 (PAR1) and 4 could give more reliable data. If so, it would correspond with studies on NOAC's. We may hypothesise that the use of TRAP as an agonist in LTA could help us to detect the effect on platelet aggregation with warfarin as well. Moreover, it is clear that the choice of a reliable and verified method is paramount because the ability to detect a change in aggregation can vary a lot (32).

We are aware of the fact that aggregability tests do not fully correspond with the global aggregation *in vivo*. What we could measure and investigate was always only one particular pathway with one concrete inducer, or one concrete receptor. The complex system of induction and suppression of aggregation allows many ways of aggregation to remain unaffected, thus precluding the detection of some particular changes in some pathways. We cannot say with certainty if the suppression in one pathway can be stronger than in others.

Conclusion

Contrary to our original hypothesis, we were not able to demonstrate the reducing effect of anticoagulation on aggregability using LTA or VerifyNow. The administration of warfarin did not produce a significant reduction in platelet aggregation. The correlation of INR with the results of aggregation induced by collagen and epinephrine turned out to be not on line with our expectations. Every increase in INR means a mild increase in aggregation of platelets, thus expressing only a minimal effect of warfarin on these pathways. The effect of warfarin on aggregation was not comprehensive; some ways of aggregation induction appeared uninfluenced.

Since the aggregability depends on the applied inducer, we speculate that the application of thrombin receptor-activating peptide (TRAP) as an agonist in LTA could eventually bring more consistent results.

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