The Veterinary Journal 203 (2015) 332–336

Contents lists available at ScienceDirect

The Veterinary Journal

journal homepage: www.elsevier.com/locate/tvjl

# Inhibition of platelet function with clopidogrel, as measured with a novel whole blood impedance aggregometer in horses

Katja A. Roscher [a,*], Klaus Failing [b], Andreas Moritz [c]

[a] Equine Clinic, Internal Medicine, Department of Veterinary Clinical Sciences, Justus-Liebig-University, Frankfurter Str. 126, 35392 Giessen, Germany
[b] Unit for Biomathematics and Data Processing, Justus-Liebig-University, Frankfurter Str. 95, 35392 Giessen, Germany
[c] Clinical Pathophysiology and Veterinary Clinical Pathology, Department of Veterinary Clinical Sciences, Justus-Liebig-University, Frankfurter Str. 126, 35392 Giessen, Germany

ARTICLE INFO

*Article history:*
Accepted 27 December 2014

*Keywords:*
Clopidogrel besylate
Equine
Multiple electrode aggregometry
MEA
Multiplate

ABSTRACT

This study aimed to validate a loading and maintenance clopidogrel dosing scheme for the inhibition of platelet function, measured by whole blood impedance aggregometry in healthy adult horses. Ten Warmblood horses received oral clopidogrel once daily. Doses were based on 50 kg weight categories and resulted in one loading dose of 6–6.5 mg/kg bodyweight and maintenance doses of 1.2–1.4 mg/kg over the next 4 days. Platelet function was measured via whole blood multiple electrode impedance aggregometry prior to (T0) and at 6, 12, 24, 48, 72, 96, 144, 192 and 240 h following the loading dose. Aggregometries for collagen (COLtest), arachidonic acid (ASPItest), adenosine diphosphate (ADPtest) and ADP with prostaglandin E1 (ADPtestHS) were performed. Statistical analyses included one way repeated measures ANOVAs and subsequent Dunnett's tests.

Platelet aggregation induced by collagen remained unchanged. There were significant inhibitions in the ASPItest ($P < 0.01$ at 192 h, and $P < 0.05$ at 240 h) and the ADPtest and ADPtestHS ($P < 0.01$, with the exception of 240 h). The loading dose of clopidogrel induced rapid inhibition of platelet function within hours, and the low dose was suitable for maintaining the inhibition over the 4 days of therapy. Recovery of platelet function was restored 6 days after the cessation of medication, determined with the ADPtest and ADPtestHS, but remained inhibited with the ASPItest. The prolonged effect of clopidogrel may indicate differences in the activation of platelets between horses and humans that were previously unknown.


## Introduction

In humans, acute systemic inflammation results in the systemic activation of the coagulation system (Yaguchi et al., 2004; Solomon et al., 2011; Levi et al., 2012), and platelet activation is widely recognised as a potent risk factor for thrombosis, especially in veins (Mavrommatis et al., 2000; Esmon, 2003). Activation of coagulation with the formation of microthrombosis and organ failure has also been demonstrated in horses (Feige et al., 2003; Cotovio et al., 2007; Armengou et al., 2008).

The role of platelet activation in systemic inflammation induced coagulopathy remains unclear. In experimental settings, platelets can be activated directly by lipopolysaccharides or the induction of endotoxaemia and may, therefore, play a role in the development of inflammatory laminitis (Weiss et al., 1997; Brooks et al., 2007; Bailey et al., 2009). Platelet activation, determined with a platelet adhesion assay or with flow cytometry, also occurs in recurrent airway obstruction (Dunkel et al., 2007) and infectious diseases (Russell et al., 1999). In systemic inflammatory disorders, the degranulation of platelets as demonstrated by decreases in mean platelet component has provided evidence of in vivo activation (Segura et al., 2007).

Common and feared consequences of systemic inflammation in horses include catheter induced venous diseases (Traub-Dargatz and Dargatz, 1994; Dolente et al., 2005), and it is highly likely that the activation of platelets is involved in this inflammatory state (Von Hundelshausen and Weber, 2007). The inhibition of platelet function may represent a valuable adjunct therapy to the routinely performed inhibition of plasmic coagulation with heparins.

In humans, inhibition of platelet function is most commonly achieved with acetylsalicylic acid, thienopyridins ($P2Y_{12}$ antagonists) or dual therapies with both substances (Bonzel et al., 2008). The most commonly used thienopyridine is clopidogrel. Horses receiving 2 mg/kg bodyweight (BW) clopidogrel were shown to exhibit a delayed inhibition commencing on the third day of therapy (Brainard et al., 2011). The dosing regimen used in humans generally comprises one loading dose of 4 to 8 mg/kg BW and subsequent daily maintenance doses of 1 to 2 mg/kg BW clopidogrel (Siller-Matula et al., 2010). With this loading dose, maximal platelet inhibition was achieved after 4 h (Müller et al., 2003).

* Corresponding author. Tel.: +49 641 9938665.
*E-mail address:* Katja.Roscher@vetmed.uni-giessen.de (K.A. Roscher).

http://dx.doi.org/10.1016/j.tvjl.2014.12.028
1090-0233/

The use of the whole blood impedance multiple electrode aggregometer (MEA) Multiplate (Roche) is well established in human platelet function testing, and the inhibitory effects of clopidogrel can be reliably and specifically detected via agonists adenosine diphosphate (ADP) and ADP in combination with prostaglandin (PG) $E_1$ (Penz et al., 2010; Chen et al., 2011). The use of this device for equine platelet function testing has recently been established (Held and Roscher, 2013) and whole blood impedance aggregometry has been applied in some studies (Jarvis and Evans, 1994; Kornreich et al., 2010).

The objective of the present study was to validate a dosing regimen involving loading and maintenance doses of clopidogrel that was designed to induce the inhibition of platelet function, as measured by whole blood impedance aggregometry in healthy adult Warmblood horses. We hypothesised that this dosing regimen would inhibit platelet aggregation within hours and maintain this inhibition over at least the next four days.

## Materials and methods

### *Study design*

Ten healthy adult Warmblood geldings ($n = 6$) and mares ($n = 4$) that had not received medication for at least the preceding 14 days were used. Health was assessed as absence of pathological abnormalities revealed by clinical examinations that included ultrasound of both jugular veins. The mean electronically determined bodyweight was 572.1 kg (range: 440–626 kg) and mean age was 15.2 years (range: 3–23 years). The horses were stabled in individual boxes with daily light exercise, fed with grass hay (1.5 kg/100 kg BW) and were provided with individualised amounts and varieties of concentrates (e.g. sugar beet, barley and corn flakes, muesli [Struktur Equichamp, Eggersmann]). Horses received clopidogrel besylate (Clopidogrel AL, Aliud) PO at 09.00 h on 5 consecutive days. Individual weight-dependent dosing was replaced by dosing in weight categories of 50 kg, which resulted in single loading doses of 6–6.5 mg/kg BW and maintenance doses of 1.2–1.4 mg/kg BW (Table 1). Hay was not withdrawn, and tablets were fed with the concentrate respectively crushed and mixed with molasses and administered via syringe by one of the authors (KR). Horses underwent a physical examination every 12 h, specifically focused on bleeding signs, reactions at venepuncture sites and drug induced adverse effects on the gastrointestinal tract.

The study was approved by the Ethics Committee for Animal Welfare of the district government of Giessen, Germany (GI 18/17-Nr. 102/2010).

### *Blood samples*

Blood samples were taken at time points (T) before (T0) and T6, 12, 24, 48, 72, 96, 144, 192 and 240 h after administration of medication. Blood was obtained by venepuncture of the jugular vein with an 18 G sterile needle and a vacuum system (S-Monovette, Sarstedt). For the measurement of platelet function tubes containing hirudin (20 μg/mL minimum final concentration, Dynabyte) were used, according to the recommendations of the manufacturer.

Laboratory examinations were completed using haematological (Advia 2120, Siemens), clinical chemistry and coagulation panels at T0, T24, T144 and T240. For these measurements, blood was obtained in tubes containing potassium-EDTA for haematology, lithium heparin for the clinical chemistry (except for the total bile acids where serum was used) and sodium citrate (0.106 M, anticoagulant to blood ratio 1:9) for the coagulation panel (all tubes S-Monovette, Sarstedt). The clinical chemistry panel included urea, creatinine, ionised electrolytes (sodium, potassium, chloride and phosphorus), total plasma protein, albumin, total bilirubin, direct bilirubin, enzymes (alkaline phosphatase, ALP; glutamate dehydrogenase, GLDH; gamma-glutamyl transpeptidase, GGT; aspartate transaminase, AST; creatine kinase, CK; lactate dehydrogenase, LDH), total bile acids (Pentra 400, Axon Lab) and ionised magnesium and calcium (Nova 8 Analyzer). Coagulation parameters included activated partial thromboplastin time (aPTT), prothrombin time (PT), PT internationalised normalisation ratio (PTINR) and fibrinogen (STA Compact, Diagnostica Stago). All measurements were completed within 60 min of blood collection.

**Table 1**
Dosing regimen for clopidogrel in weight categories with total amount (mg) and number of tablets 75 mg each ($n$) for loading and maintenance dose.

| Bodyweight | Loading dose | | Maintenance dose | |
|---|---|---|---|---|
| | Clopidogrel (mg) | Tablets 75 mg ($n$) | Clopidogrel (mg) | Tablets 75 mg ($n$) |
| 450–499 kg | 3000 | 40 | 600 | 8 |
| 500–549 kg | 3300 | 44 | 675 | 9 |
| 550–599 kg | 3600 | 48 | 750 | 10 |
| 600–649 kg | 3900 | 52 | 825 | 11 |
| 650–700 kg | 4200 | 56 | 900 | 12 |

### *Measurement of platelet function*

Platelet function was measured by whole blood impedance aggregometry (Multiplate, Roche, software version V2.03.11). The Multiplate was a five channel computerised system that used disposable cuvettes with two independent pairs of silver plated wires and a polytetrafluoroethylene coated rotating magnetic bar. The activation of platelets resulted in adhesion to the wires that lead to elevations in electrical resistance, which was continuously registered and transformed into arbitrary aggregation units and plotted against time. Two curves were assessed by the two independent sensors in each test cell. The parameters calculated by the software were the mean values of the parameters determined with each curve. Repeated measurements were advised when the coefficient of correlation was <98% or when differences in measurements of >20% of the mean were observed. The parameter that reflected the overall platelet activity was the area under the aggregation curve, which was displayed by the device in units (U).

After blood collection, tubes were stored, unagitated, at room temperature for 30 min. Aggregometry was performed according to the instructions of the manufacturer (User Manual Multiplate 5.0 Analyzer; Dynabyte GmbH, 2010). Briefly, 300 μL of blood were added to 300 μL saline (0.9%) preheated to 37 °C in the test cell. An incubation time of 180 s was initiated automatically. Aggregometry was induced by the addition of 20 μL of standardised agonists: collagen (1.6 μg/mL, COLtest, Roche), ADP (6.5 μM, ADPtest, Roche), ADP (6.5 μM) with PGE1 (9.4 nM, ADPtestHS, Roche) and arachidonic acid (0.5 mM, ASPItest, Roche). Concentrations of the agonists were used according to the recommendations of the manufacturer except that for collagen which was half of the concentration recommended by the manufacturer. Aggregation was measured over 12 min. All measurements were completed within 60 min after blood collection.

### *Statistical methods*

Statistical analyses were performed with the BMDP program package (Dixon, 1992). For the majority of variables, data descriptions represent the arithmetic mean and standard deviation (SD). After testing for deviations from the normal distribution using the normal probability plots of the residuals (Q-Q-plot), one-way repeated measures ANOVAs were performed after logarithmic respectively square root transformation of the data if required. When the ANOVAs showed significant changes over time, post hoc Dunnett's tests were used to compare each time point to T0. For each variable, the level of statistical significance was set at $P \leq 0.05$. An a priori power analysis using the program BiAS for Windows (Version 9.08) revealed a sample size of 10 horses needed to detect an expected mean reduction of 70% in platelet aggregation 24 h after the loading dose of clopidogrel with a probability of 0.95 and an accuracy of ±10%.

## Results

All horses remained clinical healthy. Clinically significant changes in haematology, clinical chemistry or coagulation panels were not detected. Platelet aggregation induced by collagen did not change at any time points. ANOVAs for ADPtest, ADPtestHS and ASPItest showed significant changes over time ($P < 0.0001$). Platelet functions were significantly inhibited ($P < 0.01$) 6 h after the administration of the loading dose. These inhibitions were maintained until T24 and for 3 subsequent days (Table 2). After cessation of clopidogrel, platelet function, as measured by ADPtest and ADPtestHS, remained significantly inhibited ($P < 0.01$) until T192. Six days after the last administration of medication (T240), the mean baseline values were recovered. Platelet function, as measured with the ASPItest, remained significantly inhibited ($P < 0.01$; T192 and T240 $P < 0.05$) until the final measurement (Table 2).

## Discussion

The loading dose of clopidogrel induced rapid inhibition of platelet function as measured by ADP-induced aggregometry within 6 h, and these inhibitions were maintained until the next administration 24 h later. The low maintenance dose was suitable for preserving this inhibition over the course of therapy. To better simulate clinical settings, the horses were not fasted before the administration of medication, and individual weight dosing was replaced by dosing based on weight categories.