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Optimization of Multiplate® whole blood platelet aggregometry in the Beagle dog and Wistar rat for *ex vivo* drug toxicity testing

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ABSTRACT

This study was performed to optimize and standardize the use of the Multiplate® whole blood impedance aggregometer in the Beagle dog and Wistar rat for use in a research laboratory environment. The anticoagulants citrate, heparin and hirudin were compared and platelet aggregation responses to ADP, collagen, arachidonic acid and Par-4 agonist were evaluated to determine their half maximal effective concentrations (EC_{50}) in blood containing low concentrations of a drug solvent (0.1% DMSO). The results indicate that citrate anticoagulation is not suitable for Multiplate® whole blood aggregometry because of the presence of spontaneous aggregation. ADP and collagen were found to be appropriate agonists for both species, whereas in the Beagle dog Par-4 agonist failed to induce aggregation and arachidonic acid induced platelet aggregation showed a high interindividual variability. The agonists EC_{50} calculated in hirudin blood were 2.70 μM ADP, 0.85 μg/ml collagen, 0.03 mM arachidonic acid and 165.7 μM Par-4 agonist in the Wistar rat, and 0.95 μM ADP and 0.23 μg/ml collagen in the Beagle dog.

1. Introduction

Drug-induced thrombosis is a well recognized life threatening condition which has been extensively documented (Ramot and Nyska, 2007) and detection of the effect that drugs can have on platelet function is one important aim in preclinical safety. Among laboratory animal species available in research, the Beagle dog and Wistar rat are commonly used as animal models for evaluation of drug toxicity (Evans, 2008). Various methods for evaluating platelet function have been described in the literature (Packham et al., 2002), and optical platelet aggregometry (also known as the "Born method") in platelet rich plasma is considered to be the gold standard for laboratory platelet function testing. In 1980, Cardinal and Flower described the impedance method for measuring platelet aggregation in whole blood instead of PRP. The main advantage of whole blood aggregometry is that the evaluation of platelet function can be performed under near physiological conditions, where platelets can interact with other blood cells. The point-of-care Multiplate® system (for multiple platelet function analyzer) was originally developed for simple and rapid impedance platelet aggregometry measurement under low, non laminar shear conditions in human whole blood (Harrison, 2009; Halimeh et al., 2010). The device has since been used for platelet function testing in animals, especially sheep and dogs (Baumgarten et al., 2009; Kalbantner et al., 2010).

Physiologically, numerous aggregating agents act in synergism to induce platelet aggregation by involving multiple adhesion receptors (Jackson, 2007). For *ex vivo* aggregation testing, commercially available agonists are often studied individually to detect specific platelet disorders and their underlying pathomechanism. Among others, ADP, collagen and arachidonic acid are well known and commonly used platelet-aggregating agents. ADP is an important physiological platelet agonist released from erythrocytes and platelets (Packham et al., 2002). ADP receptors P_2Y_{12} and P_2Y_1 are present on the platelet surface and play a role in platelet aggregation and calcium movement (Murugappan and Kunapuli, 2006). Collagen is another natural platelet agonist acting as a major aggregating agent through binding to platelet receptors glycoprotein VI and the $\alpha_2\beta_1$ integrin. Several sources of collagen are available to induce *in vitro* platelet aggregation (Soloviev et al., 1999; Takahashi, 2000) but usually fibrils from equine tendons are used. Aggregation following collagen stimulation typically displays an initial lag phase of up to a minute corresponding to the initial adhesion of platelets on collagen fibrils followed by the formation of thromboxane A_2 and release of ADP acting synergistically on the non-adherent platelets and leading to aggregation (Packham et al., 2002). The platelet agonist arachidonic acid is converted enzymatically into thromboxane A_2 which causes aggregation and release of platelet granule contents. Arachidonic acid-induced platelet aggregation is typically inhibited by aspirin. Finally, thrombin is

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the strongest aggregating agent found *in vivo* and a total of four thrombin receptors or protease-activated receptors (named Par-1 to Par-4 in order of their discovery) have been identified on platelets (Hollenberg and Saifeddine, 2001; Ramachandran and Hollenberg, 2008). Major species differences in the distribution of these receptors have been described (Rivera et al., 2009): Par-1 and Par-4 are expressed in humans, whereas Par-3 and Par-4 act coordinately on murine platelets (Ma et al., 2001). This explains why Par-1 agonists such as TRAP-6 (a commercially available Thrombin Receptor Activating Peptide) cannot be used in these species to induce platelet aggregation. TRAP-6 was previously studied in a mixed breed dog population and failed to induce platelet aggregation (Kalbantner et al., 2010). Recently, a Par-4 agonist was commercialized for platelet aggregation studies in species lacking Par-1 receptor (*i.e.* rats and dogs) (Multiplate® manufacturer data).

Anticoagulants are required for *ex vivo* platelet function testing; however their use results in a change in the blood platelet environment which could lead to a possible artificial alteration of platelet behavior (Emery and O'Dell, 1987; Solomon et al., 2008). Platelet function testing using the Multiplate® analyzer can be performed in lithium heparin, sodium citrate or recombinant hirudin blood (manufacturer data). In contrast to citrate which chelates divalent cations such as calcium and therefore requires dilution of the sample with calcium containing solutions prior to testing, hirudin and heparin preserve the level of ionized calcium in the blood sample (Glusa, 1991; Nowak and Schrör, 2007).

In previous studies, aggregation responses to several agonists have shown considerable variations between species. Moreover, strain and stock variations in the laboratory rat (Dwyer and Meyers, 1986) and variations between dog breeds in response to different agonists have been reported indicating the need for laboratory- and species-specific procedures (Kurata et al., 1995; Soloviev et al., 1999; Nielsen et al., 2007; Seyfert et al., 2007; Halimeh et al., 2010). The variability of the techniques used (optical *versus* impedance assays) combined with different anticoagulants, agonist sources and ways of determining optimal agonist concentrations impair standardization of platelet function testing in both clinical and research settings (Moffat et al., 2005; Jansinova et al., 1992).

The present study was designed to optimize and standardize the use of whole blood impedance aggregometry using the Multiplate® analyzer in the Beagle dog and Wistar rat in a research laboratory setting aiming at testing the effect of drugs solubilized in whole blood on platelet aggregation. One experimental protocol used in our laboratory is to spike blood samples with the drug in a wide range of concentrations and to run platelet function tests. Due to the poor solubility of certain drugs in whole blood, solvents such as dimethylsulfoxide (DMSO) are needed. The lowest concentration found to have the smallest effect on platelet function and used routinely in our laboratory is 0.1% (unpublished internal data). Results of platelet function tests obtained after addition of the drug are then compared to a control containing only the solvent (*i.e.* DMSO).

As our aim was to find the best conditions for testing drugs solubilized in DMSO, all aggregation measurements were performed in blood samples containing a low DMSO concentration (*i.e.* 0.1%). We first compared sodium citrate, lithium heparin and recombinant hirudin anticoagulants and then evaluated platelet aggregation responses to ADP, collagen, arachidonic acid and Par-4 agonist in order to determine their half maximal effective concentrations (EC_{50}).

2. Materials and methods

All procedures were approved by the Novartis Institutes for BioMedical Research and were performed at the Klybeck site of the Novartis Pharma AG in Basel, Switzerland. The animals did not receive any medication for 14 days prior to sampling and were healthy. The Beagle dogs (Marshall Europe, Green Hill, Montichiari (BS), Italy) were between 12 and 20 months of age and housed in an environment with controlled temperature (17–23 °C), relative humidity (35–75%) and a 12-h light/12-h dark cycle, in addition to natural daylight cycle. The dogs were kept in pens (about 2 m^2/animal) containing granulate bedding material and an elevated platform for resting. Dogs of the same gender were housed together and bitches were included in the study without consideration of their reproductive cycle. All dogs had a toy to play with and free access to balconies. They received 450 g of a certified standard dog diet dispensed by an automated feeding system from 10:00 am to 11:00 pm. Tap water from the local supply was available *ad libitum* from automatic dispensers.

The Wistar rats were supplied by Harlan laboratories B.V., Netherlands and kept in Makrolon cages (3 animals in each cage) at controlled room temperature of 20–24 °C, a relative humidity between 40% and 70% and a constant light-dark schedule (12 h artificial light from 06:00 am to 06:00 pm). The animals were fed a pellet diet (Provimi-Kliba Nr 3893) and fresh sterile water *ad libitum*. The rats mean body weight was 450 g.

2.1. Blood sampling techniques

2.1.1. Beagle dog

Jugular venipuncture was performed after minimal manual compression of the vein using a 20G needle for S-Monovette (Sarstedt). Blood was collected into either 2.6 ml S-Monovette hirudin tubes (Sarstedt), 2.9 ml S-Monovette trisodium citrate tubes (Sarstedt) or 4.5 ml S-Monovette lithium heparin tubes (Sarstedt).

2.1.2. Wistar rat

Blood was taken after CO_2 euthanasia by cardiac puncture and punction of the caudal *vena cava* using a 20G needle connected to a Multi-adapter (Sarstedt). Blood was anticoagulated either with hirudin (S-Monovette, 2.6 ml, Sarstedt), trisodium citrate (S-Monovette, 1.4 ml, Sarstedt) or lithium heparin (S-Monovette, 1.2 ml, Sarstedt).

2.2. Multiplate® measurements

Aggregation measurements were performed between 30 min and 2 h post-bleeding. All blood samples were incubated 10 min with DMSO (Sigma–Aldrich Chemie, GmbH, Seinheim, Germany) at a final concentration of 0.1% on the plate agitator Titramax100® at speed 1.5. Reconstitution of the agonists (ADPtest, COLtest, ASPItest and Par-4 Agonist, Dynabyte GmbH, Munich, Germany) was done as specified by the manufacturer. The agonists were diluted to their final concentrations using isotonic sterile NaCl solution and stored at 4 °C between runs for a maximum of 24 h. Whole blood platelet aggregation was performed according to the manufacturer's instructions on two Multiplate® analyzers with the Multiplate® software version V2-03. Electronic controls were run daily before aggregation measurements were done. Briefly, hirudin and heparin samples were diluted 1:2 in an isotonic NaCl solution and citrate samples in a $NaCl/CaCl_2$ solution (3 mM) (Dynabyte GmbH, Munich, Germany). After a 3-min incubation time and stirring at 37 °C, platelet aggregation was triggered by adding one agonist solution. Platelet aggregation measurements for rat samples were performed as described above except that Multiplate® Mini Test Cells were used, which allow the use of small blood volumes (175 μl). Control measurements for spontaneous aggregation were performed by replacing the agonist volume with isotonic NaCl.