Pesticide Biochemistry and Physiology 146 (2018) 19–24

Contents lists available at ScienceDirect

Pesticide Biochemistry and Physiology

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

Thrombin generation test: A reliable tool to evaluate the pharmacodynamics of vitamin K antagonist rodenticides in rats

Jourdi Georges[a,1], Lefèbvre Sebastien[b,1], Le Bonniec Bernard[a], Curis Emmanuel[c], Gaussem Pascale[d], Lattard Virginie[b,2], Siguret Virginie[e,*,2]

[a] INSERM UMR_S1140, Université Paris Descartes, Sorbonne Paris Cité, 4 avenue de l'Observatoire, 75006 Paris, France
[b] USC 1233 RS2GP, VetAgro Sup, INRA, Univ Lyon, F-69280, 1, avenue Bourgelat, 69280 Marcy l'Etoile, Lyon, France
[c] Laboratoire de biomathématiques & UMR_S1144, Université Paris Descartes, Sorbonne Paris Cité & DBIM, Hôpital Saint-Louis, AP-HP, 4 avenue de l'Observatoire, 75006 Paris, France
[d] Service d'hématologie biologique, Hôpital Européen Georges Pompidou, AP-HP & INSERM UMR_S1140, Université Paris Descartes, Sorbonne Paris Cité, 4 avenue de l'Observatoire, 75006 Paris, France
[e] Service d'hématologie biologique, Hôpital Lariboisière, AP-HP & INSERM UMR_S1140, Université Paris Descartes, Sorbonne Paris Cité, 4 avenue de l'Observatoire, 75006 Paris, France

ABSTRACT

Vitamin K antagonist rodenticide pharmacodynamics (PD) is studied in rodents with traditional laboratory tests. We wondered if thrombin generation test (TGT) could add value.

Difethialone (10 mg/kg) was administered *per os* to 97 OFA-Sprague Dawley rats. PD was studied over a 72 h-period using the Calibrated Automated Thrombogram on platelet poor plasma before and after intoxication (3 female and 3 male rats for each 13 time points) and TGT parameters were compared with the prothrombin time (PT) and vitamin K dependent factor activities previously reported.

Following intoxication, preliminary tests evidenced rapid and full inhibition of thrombin generation triggered with 5 or 20 pM human recombinant tissue factor. To study the evolution of TGT parameters following difethialone intake, we adapted the test by complementing intoxicated rat samples with pooled normal rat plasma (3/1, v/v). Adapted TGT confirmed the known higher procoagulant basal level in females compared to males through higher endogenous thrombin potential (ETP) and peak height (PH) ($p < 0.0001$ and $p = 0.0003$, respectively). An exponential model fitted well the PH and ETP decay after intoxication. In contrast to PT, the decreases were observed immediately following VKA intake and had comparable time to halving values: 10.5 h (95% CI [8.2; 13.6]) for ETP and 10.4 h (95% CI [7.8; 14.1]) for PH. The decrease of FVII and FX preceded that of PH, ETP and FII while FIX decreased later on, contributing to the severe hypo-coagulability.

We demonstrated that TGT performed in samples of intoxicated rats complemented with normal plasma is a reliable tool for evaluation of VKA rodenticide PD in rats.

1. Introduction

Vitamin K antagonists (VKA) have been used as rodenticides for more than sixty years [1]. They exert their anticoagulant effect by targeting the vitamin K 2,3-epoxide reductase complex subunit 1, therefore blocking the recycling of vitamin K 2,3-epoxide in vitamin K hydroquinone [2]. Decreased availability of vitamin K hydroquinone, a co-substrate of the γ-carboxylase, results in hypo-γ-carboxylated vitamin K dependent clotting factors thus in dramatically decreased clotting activity of factors II, VII, IX and X (FII, FVII, FIX, FX) [3,4].

VKA rodenticides are characterized by a wide inter-individual variability of response between rats due to gender, environmental and genetic factors [1,5,6]. The origin of the gender differences in the response to VKA rodenticides has been reported in different rat strains: in

Abbreviations: CAT, calibrated automated test; ETP, endogenous thrombin potential; F, factor; LT, lag time; PD, pharmacodynamics; PH, peak height; PNrP, pooled normal rat plasma; PPP, platelet poor plasma; PT, prothrombin time; TGT, thrombin generation test; TTP, time to peak; VKA, vitamin K antagonist

* Corresponding author at: UMR_S1140, Faculté de Pharmacie, 4 avenue de l'Observatoire, 75006 Paris, France.
E-mail addresses: georges.el-jerdi@parisdescartes.fr (J. Georges), sebastien.lefebvre@vetagro-sup.fr (L. Sebastien), bernard.le-bonniec@parisdescartes.fr (L.B. Bernard), emmanuel.curis@parisdescartes.fr (C. Emmanuel), pascale.gaussem@parisdescartes.fr (G. Pascale), virginie.lattard@vetagro-sup.fr (L. Virginie), virginie.siguret@parisdescartes.fr (S. Virginie).
[1] These authors share equal contribution to the work.
[2] These authors share equal senior authorship.

https://doi.org/10.1016/j.pestbp.2018.02.004
Received 17 June 2017; Received in revised form 1 February 2018; Accepted 14 February 2018
Available online 15 February 2018

Sprague-Dawley rats, a higher basal activity of FVII and FX has been found in females compared to males, in agreement with results of older studies in Wistar rats [6,7]. Besides, resistance to VKA rodenticides due to genetic factors has been reported in both genders from many countries in Europe but also in United States, Canada, Japan and Australia resulting in a serious concern for pest control [8–10]. Consequently, warfarin and diphacinone which had been introduced in the early 1950s, were replaced in the 70s by more potent molecules such as difenacoum or bromadiolone, and 10 years later by brodifacoum or flocoumafen. Nevertheless, genetic resistance to these compounds developed in parallel [1,5,11–14]. Difethialone was the latest VKA rodenticide developed for pest control and is currently considered as one of the most powerful for which no resistance has been described so far [15].

Published data on the rodent PD profile of VKA rodenticides are scarce and report results obtained using coagulation tests of human clinical practice performed with rodent plasma samples [5,7,16,17]. Semi-global coagulation assays, namely prothrombin time (PT) and activated partial thromboplastin time are reliable tools for the exploration of coagulation system in rodents, especially to assess the anticoagulant resistance [18]. However, they mainly explore the initiation phase of coagulation, which requires a minimal amount of thrombin generated (*i.e.* 10–30 nM) necessary for clot formation [19]. More recently, additional global tests, among which the thromboelastometry and the thrombin generation test (TGT), have been proposed to evaluate thrombin generation beyond clot formation [20–22], thus to follow rodents anticoagulation by VKA and propose a better rodenticides management. Rotational thromboelastometry (Rotem) did not provide a very good insight of coagulability in this setting since only two parameters obtained with Extem were modified in intoxicated rat Platelet Poor Plasma (PPP) with lag time higher than 8 h and very wide confidence ranges of their doubling time along with the relatively large sample volume needed. Consequently, PT remains the best method to monitor VKA rodenticides induced hypocoagulation [22]. In TGT, the addition into plasma of a fluorescent substrate cleaved by thrombin allows measuring a fluorescence signal over time, which is not disturbed by the turbidity of a forming clot, in contrast to optical density for a chromogenic substrate [23]. In humans, TGT accurately evaluates coagulation disorders associated with bleeding or thrombosis. For example, TGT provides another insight on anticoagulation improving the monitoring of factor replacement therapy in hemophilic patients beyond clotting times [24–27]. However, this method has never been developed in rats intoxicated with VKA rodenticide in order to evaluate their impact on the coagulation system and therefore the rodenticide efficacy.

In a recent study, our group determined the pharmacokinetic/pharmacodynamic profile of difethialone in male and female Sprague-Dawley rats over a 72-hour period following difethialone intoxication using routine coagulation tests [7]. The aim of the present study was (i) to adapt the standard Calibrated Automated Thrombography (CAT) protocol to ensure the reliability of TGT to evaluate anticoagulant rodenticide PD in rat plasma samples and (ii) to determine the kinetic profiles of TGT parameters from individual intoxicated male or female rats and built models of TGT parameters kinetics after VKA intoxication. Finally, we compared TGT parameter decays to those of PT and vitamin K dependent clotting factor activities.

2. Materials and methods

2.1. Animals and blood sampling

All research procedures were performed according to an experimental protocol following international guidelines (86/609/EEC) and approved by the ethic committee of VetAgro Sup. A total of 97 eight-week old male and female OFA-Sprague-Dawley rats (Charles River, l'Arbresles, France) were used in the present study. All rats were acclimatized for at least five days and housed in the animal facility, four rats per cage as already reported in details [7]. Just before blood sampling, rats were anesthetized with isoflurane and euthanized with CO_2 immediately after sampling.

2.1.1. Blood sampling and preparation of pooled normal rat plasma (PNrP)

Since female have higher basal procoagulant level compared to male rats [7], we chose to use only male rats to prepare a pool of normal rat plasma. Therefore a group of 10 male rats were fed with standard diet Scientific Animal Food and Engineering, referenced A04 (Augey, France). Blood was collected by cardiac puncture in 3.5 mL tubes containing sodium citrate (3.2% 0.105 M; 1:9 v/v; Greiner, Alcyon, Nancy, France) and gently inverted five times to ensure adequate anticoagulation. Tubes were then double centrifuged at 2000*g* 15 min at 20 °C and PPP samples were pooled and served as Pooled Normal rat Plasma (PNrP). PNrP was aliquoted, frozen and stored at −80 °C until use. PT and aPTT results measured on PNrP were in the reference intervals [7].

2.1.2. Blood sampling and preparation of PPP from intoxicated rats

In order to adapt the CAT protocol for intoxicated rat PPP samples, 9 male rats were fed with vitamin K3-deficient food (Scientific Animal Food and Engineering), at least 48 h before the beginning and throughout the experience as described [7]. Animals received by force-feeding a *per-os* administration of 10 mg/kg difethialone (kindly provided by Liphatech, Pont-du-Casse, France), corresponding to 20-fold the lethal dose LD50. Pooled PPP samples from 3 male rats obtained at T0, T24 and T72 after difethialone intoxication were prepared and stored as described above.

In a second phase and in order to study coagulation system after intoxication with difethialone, 39 male and 39 female rats were force-fed as above. Groups of 3 males and 3 females were anesthetized prior to blood sampling as described above at different times (T) (in hours) after difethialone administration: T0, T1, T2, T4, T8, T10, T13, T16, T20, T24, T32, T48 and T72. All PPP samples were thawed in a 37 °C water bath just before use.

2.2. Thrombin generation test in rat PPP

2.2.1. CAT assay

TGTs were performed on a CAT system according to the method described by Hemker et al. [23]. TGTs were measured using a microplate fluorometer (Fluoroskan Ascent™, ThermoLabsystems, Helsinki, Finland). Rodent PPP tends to have short lag time and rapid thrombin generation resulting that if all of the 96 wells of a microplate are used simultaneously, lack of the early points for the second half of the microplate precludes accurate ETP evaluation [28]. Using only the first half of the 96-well plate allows starting on time all fluorescence measurements following initiation. Consequently, half of a round bottom 96-well plate (Greiner, Frickenhausen, Germany) was used each time. PPP samples were run in duplicate using either 20 µL PPP-reagent (5 pM tissue factor and 4 µM phospholipid vesicles; Stago, Asnières sur Seine, France) or 20 µL High PPP-reagent (20 pM tissue factor and 4 µM phospholipid vesicles; Stago), and 20 µL Thrombin Calibrator (Stago) in parallel; average of the duplicates was used for analysis and mean CV values varied between 1.5% for temporal TGT parameters and 3% for the others. TGT plate containing the mixture of 80 µL PPP and 20 µL triggering reagent or Thrombin Calibrator was incubated 5 min in the machine to warm to 37 °C. TGT was initiated by autodispensing 20 µL of FluCa solution (Stago) containing calcium ions and the fluorogenic substrate, Z-Gly-Gly-Arg-7-amino-4-methylcoumarin, to each well. Generated thrombin cleaves the fluorogenic substrate and fluorescence increase was monitored at 37 °C for 120 min. Thrombinoscope software (Thrombinoscope® BV, version 5.0.0.742, Maastricht, Netherlands) was used to calculate the amount of thrombin generated over time expressed as human thrombin equivalent taking into account for each