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Strain differences in intestinal toxicity of warfarin in rats

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ABSTRACT

Intestinal hemorrhage characterizes effectiveness of warfarin (WF) as rodenticide and is among adverse effects of therapy in humans. Having in mind genetic variations in the effectiveness of WF in wild rats and in the doses required for therapeutic effect, strain differences in the intestinal toxicity of oral warfarin in rats were examined in this study. High WF dose (3.5 mg/l) led to mortality in Albino Oxford (AO) rats, with no lethality in Dark Agouti (DA) rats. Higher values of prothrombin time were noted at low WF dose (0.35 mg/l) in the former strain. Leukocyte infiltration in intestine noted at this dose in both strains was associated with oxidative injury and more pronounced anti-oxidative response in AO rats. Suppression of mesenteric lymph node cell proliferation and IFN-γ and IL-10 production in AO rats and lack of these effects in DA rats, represent different strategies to protect vulnerable intestine from harmful immune responses.

1. Introduction

Warfarin (4-OH coumarin) and its congeners are extensively used in the control of pest rodents. Effects of these chemical agents as rodenticides are based on their action as vitamin K (VK) antagonists which inhibit vitamin K-dependent (VKD) step in liver synthesis of blood coagulation factors (F) including FII (prothrombin, PT), FVII, FIX and FX which are required for normal blood coagulation (Shearer, 1990). Warfarin affects interconversion of vitamin K and its 2,3-epoxide (VKO) *via* inhibition of vitamin K epoxide reductase (VKOR). Rapid depletion of hydroquinone (K_1H_2), a cofactor of γ-glutamyl carboxylase which mediates generation of biologically active proteins involved in coagulation process by carboxylation of glutamyl (Gla) residues on precursors of several VKD, takes place as a consequence of VKOR inhibition (Furie, 2000). Depletion of these factors results in an increase in time necessary for blood to clot, up to the point where no clotting occurs. Concomitant injury of small blood vessels, which might occur, results in death of the animal from internal hemorrhage (Lund, 1988). Anticoagulation with warfarin is also used to prevent thromboembolic diseases in patients at risk (Furie, 2000).

Over years, some populations of wild rats in urban habitats turned out resistant to warfarin action in terms of very low anticoagulant effect (Ishizuka et al., 2007; Lasseur et al., 2005; Thijssen et al., 1989). Variations in warfarin dose which is required to achieve a therapeutic effect (e.g. 20-fold) were reported in patients as well (Wadelius et al., 2007).

Studies with warfarin-resistant rats (bred in the laboratory from wild rats initially trapped outdoor and maintained in outdoor enclosures) revealed that VKOR, pharmacological target of warfarin, is weakly inhibited by this anticoagulant in resistant rats and that basal activity of this enzyme is frequently lower in resistant animals (Thijssen et al., 1989). Warfarin resistance is attributable to mutations in the subunit 1 of vitamin K1 epoxide reductase complex (*VKORC1*) gene (Lasseur et al., 2005; Pelz et al., 2005; Rost et al., 2004). Data from other studies suggest that mutations in *VKORC1* alone cannot explain all aspects of resistance in rats (Wajih et al., 2004) and that variations in anticoagulant uptake,

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metabolism, and clearance may influence the efficacy of rodenticides *in vivo*. Warfarin-resistant rats had high metabolic activity of P450, which might be due to the high expression of CYP3A and elevated activity of NADPH-P450 reductase (Ishizuka et al., 2007). Human studies showed variability in genes for proteins involved in the effect of and biotransformation of warfarin and that linked single nucleotide polymorphisms of *VKORC1* and *CYP2C* genes were the strongest genetic factors determining warfarin dose requirements in patients (Wadelius et al., 2007). Other authors suggest up-regulation of compensatory mechanisms aimed in regeneration of hydroxyvitamin K which can help to maintain the activity of coagulation factors (Wallin et al., 2001). The proposed model provided molecular mechanism for warfarin resistance which is based on the effects of the vitamin K-dependent γ-carboxylation system in the rat and is applicable to resistance to this agent in some patients on anticoagulant therapy.

Beside proteins involved in process of blood coagulation, vitamin K-dependent (VKD) γ-carboxylation system is involved in generation of bioactive VKD proteins produced by extrahepatic tissues including osteocalcin (OC) and matrix Gla protein (MGP) (Price, 1988), periostin 1 (Coutu et al., 2008), Gas6 protein (Nakano et al., 1997; Yanagita et al., 1999), and Gla-rich protein (Viegas et al., 2008). Inhibition of MGP by warfarin is considered responsible for developmental derangements in pregnant women on therapy with this agent ("warfarin embryopathy") (WHO, 1995) and for arterial wall calcification in patients on warfarin therapy (Chatrou et al., 2012). Warfarin effects on nuclear receptor binding vitamin K (Pxr) were suggested as responsible for developmental toxicity in aquatic organisms (Fernández et al., 2014; Weigt et al., 2012) which stressed the importance of warfarin as environmental contaminant and raised concerns about human health. However, there are no data concerning variations in the effect of warfarin on VKD proteins and the process not related to hemostasis.

Beside impact on VKD proteins and related processes, warfarin exerts effects on other physiological processes including reactions of immune defense. Both suppressive and stimulating effects of warfarin on components of immune system were described in humans and laboratory animals. Inhibition of skin induration in delayed hypersensitivity (DTH) tests and down regulation of DTH reaction were described in humans on warfarin therapy and in rats, respectively (Edwards and Rickles, 1978; Eichbaum et al., 1979). Inhibition of inflammation in lungs (Perez et al., 1994) and pancreas (Kurohara et al., 2008) was reported in warfarin-treated mice. Increase in natural killer (NK) cell activity in patients on warfarin therapy (Bobek et al., 2005) explain the use of this agent as an adjuvant antitumor therapy in humans (Bobek and Kovarik, 2004). Studies in mice, however, showed tumorigenic potential of warfarin in mice exposed to high doses of this agent (Lake et al., 1994). Some clinical complications of warfarin therapy expressed as infiltration of inflammatory cells in affected tissues (Jo et al., 2011; Kapoor and Bekaii-Saab, 2008) imply inflammatory potential of this agent. Similarly to scarcity of data concerning the variations in effects of warfarin on VKD proteins and the process not related to hemostasis, no known variations in immune-based host cell reactivity to warfarin have been reported. Our recent data showed strain-related differences in the effect of oral warfarin intake on the peripheral blood leukocyte activity in rats (Djokić et al., 2013) which implies that there might be variations in immunomodulatory effects of warfarin.

Using the model of subacute (30 day) oral intake of warfarin in laboratory rats it was showed that it affected intestine, the tissue which was under direct influence of this agent (Mirkov et al., 2016). Regarding these and the above cited findings, the aim of the present paper was to examine whether there were strain differences in the intestinal toxicity of warfarin. To this aim, the response of gut to oral warfarin administration was examined in Albino Oxford (AO) rats and compared with the effects in Dark Agouti (DA) rats. These two strains were chosen because of their differences in immune-mediated susceptibility to insults in variety of tissues including nervous system (Lukic et al., 2001), gut and joints (Kovačević-Jovanović et al., 2015), skin (Popov Aleksandrov et al., 2015) and lungs (Mirkov et al., 2015). The effects analyzed were selected on the basis of our recent data which showed that administration of warfarin in doses which, after extrapolation, corresponded to those most often used in humans, affect intestinal immune system of DA rats (Mirkov et al., 2016). Data from the present study showed that AO and DA rats differed in the susceptibility to warfarin-induced hemorrhage, in the direct toxicity of warfarin to intestine and in the strategy to prevent potentially harmful immune reactions in gut-draining lymph nodes. These results show that beside genetically-based differences in anticoagulant effect, variations in the responses to warfarin-induced immune-mediated tissue toxicity exist, which are novel findings.

2. Materials and methods

2.1. Chemicals

Warfarin sodium (CAS number 129-06-06) was purchased from Serva Fein Biochemica (Heidelberg, Germany), purity > 98%. Phenylmethanesulfonyl fluoride (PMSF), 3-(4,5-dimethyl-thiazol-2-yl)-2,5 diphenyl-tetrazolium bromide (MTT), Concanavalin A (ConA), 2-thiobarbituric acid, trichloroacetic acid, malondyaldehide and L- epinephrine were purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA). Hydrogen peroxide (H_2O_2) was purchased from Zorka Farma Sabac, Serbia. All solutions for cell culture experiments were either prepared under sterile conditions or were sterile filtered (Flowpore, pore size 0.22 μm) before use. Culture medium RPMI-1640 (Biowest, Nuaillé, France) supplemented with 2 mM glutamine, 20 μg/ml gentamycine (Galenika a.d., Belgrade, Serbia), 5% (v/v) heat inactivated fetal calf serum (Biowest, Nuaillé, France) was used in cell culture experiments.

2.2. Animals and warfarin treatment

Animal treatment and experimental procedures were carried out in compliance with the Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes and were approved by the Ethical Committee of the Institute for Biological Research 'Sinisa Stankovic' (IBISS), University of Belgrade, Serbia. Male Albino Oxford (AO) and Dark Agouti (DA) rats, 12–14 weeks old, conventionally housed at IBISS, were used in experiments. Warfarin sodium solution was prepared in drinking water at concentration of 0.35 mg/l and 3.5 mg/l and was given to rats for 30 days. Control rats were given drinking water solely. Warfarin and water were replaced with freshly prepared solution or water twice a week. For survival study animals were inspected daily. All functional measurements were carried out at day 30 of the treatment period in animals anesthetized by *i.p.* administration of 40 mg/kg b.w. of thiopental sodium (Rotexmedica, Tritau, Germany).

2.3. Clinical biochemistry

Prothrombin time (PT) was determined in blood samples diluted in citrate buffer (1:5) by one-stage method using citrate plasma and Thromborel S reagents (Behring Diagnostics GmbH, Marburg, Germany) with Siemens equipment.

Total leukocyte counts were determined by improved Neubauer hemocytometer. Differential leukocyte counts were determined by differentiating at least 300 cells from air-dried whole blood smears stained according to the May-Grünwald-Giemsa (MGG) protocol.