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GRANULOCYTE-STIMULATING ACTIVITY OF THE ANTICOAGULANT WARFARIN IN RATS.

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Warfarin (4-OH coumarin) and its congeners are anticoagulants whose biological activity is based on vitamin K (vit. K) antagonism and subsequent inhibition of the vitamin K-dependent (VKD) step in the complete synthesis of a number of blood coagulation factors required for normal hemostasis (Shearer, 1990). Based on these activities, hydroxycoumarin anticoagulants have found wide application as anticoagulant rodenticides (Lund, 1988) and in prophylactic medicine to prevent thromboembolic disorders for more than 50 years (Furie, 2000). Warfarin (WF) and its analogs inhibit vit. K epoxide reductase (VKOR), leading to depletion of the hydroquinone form of vit. K, a cofactor for γ-glutamyl carboxylase which mediates post-translational modification (carboxylation) of nascent glutamyl (Gla) residues needed for biological activity of coagulation factors (Wallin and Hutson, 2004). Prevention of normal production of these essential blood clotting factors by hydroxycoumarin anticoagulants leads to increase of clotting time up to the point where no clotting occurs. By inhibiting VKOR, however, WF affects the generation of biologically active VKD proteins required for biological processes other than hemostasis, including proteins involved in the regulation of bone growth and calcification (bone Gla protein, BGP/osteocalcin and matrix Gla protein, MGP) (Price, 1988) and proteins involved in mesangial and vascular smooth muscle cell growth and signaling (Nakano et al., 1997; Yanagita et al., 1999). Data which demonstrated WF effects on proteins not dependent on vit. K (non-VKD proteins), including molecules involved in signal transduction (Kater et al., 2002), and the discovery of VKD proteins with as yet unknown function (Shearer, 2006) imply broader effects of hydroxycoumarin-type anticoagulant rodenticides on cell/tissue physiology.

Intensive application of anticoagulant rodenticides as a means of controlling pest rodents has raised concern about possible exposure of both humans and non-target animals to these agents. In cases of incidents of WF intoxication, systemic effects (coagulopathies, occasionally with fatal outcome) were mainly described (WHO, 1995). Clinical studies revealed cases of adverse effects of WF therapy including embryopathy and loss of bone mass in patients on prolonged therapy (WHO, 1995) as well as various other side effects, including skin necrosis (Hermes et al., 1997). In 1995, the World Health Organization Environmental Health Criteria (EHC) Program categorized WF and other hydroxycoumarins as highly dangerous agents (WHO, 1995), highlighting the need for information about their biological effects.

Along with traditional toxicity studies, immunotoxicity investigation is a recommended approach in examining the adverse effects of chemicals (WHO, 1996). Our previous study demonstrated an immunotoxic capacity of WF in the form of suppression of cells of the lymphoid compartment in an experimental model of the contact hypersensitivity reaction in laboratory rats (Kataranovski et al., 2003). To obtain initial information concerning the immunotoxicity of WF on innate immunity of cells, the effect of WF administration *in vivo* on granulocytes in rats was assessed. Quantitative changes (total and differential cell numbers) and the activation status of peripheral blood granulocytes and granulocytes migrating into subcutaneously implanted sponges containing WF were analyzed. The obtained data were obtained which suggest peripheral blood granulocyte activation following exposure to WF *in vivo*.

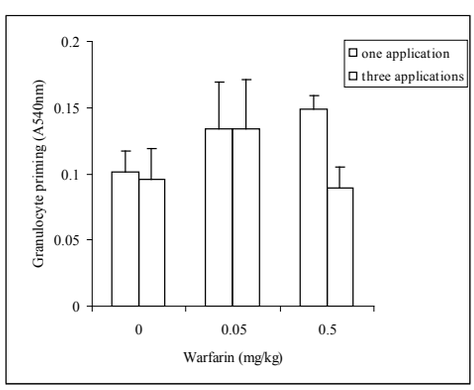

Fig.1. Peripheral blood granulocyte activity following WF application (* p<0.05 vs control WF 0).

Warfarin-sodium, WF (Serva, FR Germany) dissolved in nonpyrogenic saline was applied intraperitoneally (*i.p.*) to male Albino Oxford (AO) rats in doses of 0.05 and 0.5 mg/kg body mass for one day (1x) and for three consecutive days (3x). WF bioactivity was assessed by measuring prothrombin (PT) time (in seconds) in the blood of treated rats, employing a one-stage

method with citrate blood plasma and thromborel S (Kurata and Horii, 2004). Total and differential blood leukocyte counts were performed on whole blood smears stained by the May-Grunwald-Giemsa method. Peripheral blood granulocytes were isolated 24 hours after the last application by dextran sedimentation and density-gradient centrifugation as described previously (Kataranovski et al., 2003). Activity of granulocites was evaluated by measuring their responsiveness to exogenous stimulation (granulocyte priming). A cytochemical assay for the respiratory burst of granulocytes (Monboisse et al., 1991), based on their phorbol-12-myristate-13-acetate (PMA)-stimulated ability to reduce nitroblue tetrazolium salt (NBT), was used. The direct effect of WF on granulocytes was examined using the subcutaneously-implanted sterile polyvinyl sponge model (Pejnović et al., 1995) in a modification by which granulocytes migrate into sponges soaked in WF solution. One day after sponge implantation, migrating cells were isolated and their composition and activation status analyzed as described for peripheral blood granulocytes. Results are expressed as mean values ± standard deviation (SD) for each experimental group in *ex vivo* evaluation or as means ± standard errors (SE) for triplicates or quadruplicates from one representative experiment out of three in the case of *in vitro* measurements. Significance was defined by Student's t-test, p values of less than 0.05 being considered significant.

Intraperitoneal injection of the higher dose of WF resulted in increase of prothrombin (PT) time both after one application (122.3±10.9 s, $p<0.001$ *vs* control 27.66±9.26 s) and following three applications (126.5±6.3 s, $p<0.001$ *vs* control 26.7±4.3 s), demonstrating a basic biological effect of WF. One administration of 0.05 mg of WF/kg or 0.5 mg of WF/kg resulted in no changes in total and differential leukocyte numbers (Table 1). Administration of the lower dose of WF in a regime of three consecutive days resulted in increase in relative numbers of peripheral blood granulocytes and decrease in relative numbers of lymphocytes. Three-time application of the higher WF dose led to decrease in total leukocyte numbers. The massive internal hemorrhaging (with fatal outcome in 10% of the animals) noted at this regime of WF administration might account for the observed decrease. Administration of one lower or higher WF dose, as well as administration of the lower WF dose for three consecutive days resulted in an increase of granulocyte responsiveness to exogenous (PMA) stimulation. No changes were noted following application of the higher WF dose. The absence of a priming effect of WF might have resulted from its toxicity in this regime of application, as quantitative data (decrease in total leukocyte numbers) suggest. Given the priming effect of WF administration on peripheral blood granulocytes, the direct effect of WF exposure on granulocytes was examined. No changes in numbers of cells migrating into WF-containing sponges or their priming status were noted (not shown). The absence of a direct effect of WF on sponge-granulocytes implies the need for metabolically processed WF for the observed granulocyte-priming activity and/or contribution of the systemic environment (peripheral blood) to activation of these cells.

In conclusion, results presented in this paper demonstrated proinflammatory activity of warfarin administration. Along with our previous report regarding the immunosuppressive effects of warfarin, the presented results are a source of new data concerning the immunity-modulating potential of warfarin.

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Table 1. *Peripheral blood leukocyte numbers in rats following warfarin administration*

Warfarin dose (mg/kg b.m.)	0		0.05		0.5	
	1x	3x	1x	3x	1x	3x
Total leukocytes ($x10^9$/L)[a]	6.03±1.23	5.57±0.79	8.35±2.85	5.90±1.58	4.67±0.093	3.20±1.07*
Neutrophils (%)	27.00±8.20	20.46±2.86	27.89±4.67	30.15±6.85 *	29.51±3.2	23.39±3.27
Lymphocytes (%)	72.10±7.60	79.50±2.50	71.92±4.76	68.99±6.50*	70.17±3.34	76.30±3.15

[a] Data represent means ± SD from 4-6 animals. * $p<0.05$ *vs* control (WF 0).

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