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# STRAIN DIFFERENCES IN INTESTINAL TOXICITY OF WARFARIN IN RATS

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**Highlights**

- Intestinal toxicity of oral warfarin was examined comparatively in AO and DA rats
- High warfarin (WF) dose (3.5 mg/l) resulted in lethality in more than 80% of AO rats
- Higher values of prothrombin time were noted at low WF dose (0.35 mg/l) in AO rats
- Intestinal oxidative injury by low WF was noted in AO rats
- Suppression of mesenteric lymph node cell activity was noted only in AO 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.

**Keywords**: Oral warfarin administration; Albino Oxford (AO) and Dark agouti (DA) rats; Intestinal injury; Mesenteric lymph nodes.

## 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

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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 et al., 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, 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

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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 et al., 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 (Fernandez 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

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down regulation of DTH reaction were described in humans on warfarin therapy and in rats, respectively (Edwarsd 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 (Djokic 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

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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.

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### *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.

### *2.4. Histology*

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Samples of duodenum and jejunum were collected at necropsy, fixed in 4 % formaldehyde (pH 6.9) and processed for embedding in paraffin wax for subsequent sectioning at 5 μm. Hematoxylin and eosin (H&E)-stained tissue sections were analyzed by a certified histopathologist in blinded manner using a Coolscope digital light microscope (Nikon Co, Tokyo, Japan).

### *2.5. Preparation of intestinal homogenates*

Segments of duodenum were removed, washed through the lumen with ice-cold non-pyrogenic physiological saline and cut through. After weighing tissue was snap frozen in liquid nitrogen and stored at -80°C until use. Tissue samples were homogenized by IKA T18 Basic Homogenizer (IKA Works Inc, Wilmington NC, USA) in ten volumes of sucrose buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA, 250 mM sucrose) containing 1 mM phenylmethanesulfonyl fluoride (PMSF), on ice. Antioxidant enzyme activity and malondyaledhide were determined in supernatants obtained following ultra-centrifugation at 100,000 × g for 1 h and 45 min, at 4°C.

### *2.6. Superoxide dismutase (SOD, EC 1.15.1.1) and catalase (CAT, EC 1.11.1.6) activity in intestinal homogenates*

Activity of SOD was determined by the epinephrine method (Misra and Fridovich, 1972). One unit of activity was defined as the amount of enzyme necessary to decrease the rate of epinephrine auto-oxidation by 50% at pH 10.2. CAT activity was determined by the rate of $H_2O_2$ decomposition measured spectrophotometrically at 240 nm as described (Beutler, 1982). One unit of CAT activity was defined as the amount of enzyme that decomposed 1 mmol $H_2O_2$ per minute at 25 °C and pH 7.0. The measurements of SOD and CAT activities were accomplished

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using Shimadzu UV-1800 spectrophotometer (Kyoto, Japan). Protein concentration was determined by Lowry assay (Lowry et al., 1951) using bovine serum albumin (Fraction V obtained from Sigma-Aldrich, St. Louis, MO, USA) as a reference. Changes in SOD and CAT are expressed as relative changes, calculated as percentages of the value obtained in control (warfarin 0 mg/l) animals, which were considered as 100%.

***2.7. Malondialdehyde (MDA) in intestinal homogenates***

Lipid peroxidation was measured as malondialdehyde (MDA) production and assayed by the thiobarbituric acid reaction as described (Villacara et al., 1989). Briefly, tissue homogenates were fixed with thiobarbituric acid-trichloroacetic acid (TCA) reagent and Tris-Cl (pH 7.4) and heated for 60 min at 100°C. The absorbance of the supernatant obtained by centrifugation was measured at 535 nm using spectrophotometer (Shimadzu Corporation, Lakewood, USA). Changes in MDA were expressed as relative changes, calculated as percentages of the values obtained in control (warfarin 0 mg/l) animals, which were considered as 100%.

***2.8. Mesenteric lymph node cell preparation***

Mesenteric lymph node (MLN) cell suspensions were prepared by mechanical teasing of tissue over nylon mesh (70 μm nylon, BD Bioscience, Bedford, USA). Cells were counted by the improved Neubauer hemocytometer. Cell viability was determined by trypan blue exclusion assay and by quantitative colorimetric MTT assay for cell's metabolical viability based on reduction of tetrazolium salt MTT by mitochondrial dehydrogenases to colored end product, formazan (Oez et al., 1990). MTT test was used to measure lymph node cells metabolic viability *ex vivo* and following 48 h culture to access cell survival.

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### *2.9. Mesenteric lymph node cell proliferation*

For proliferation measurements, MLN cells ($0.5 \times 10^6$/well) were cultured in medium solely (non-stimulated proliferation) or in the presence of 2.5 μg/ml of ConA (stimulated proliferation). After 48 h, 0.5 μCi [$^3$H]-thymidine (GE Healthcare, UK) per well was added and incubated for additional 16 h. Incorporation of [$^3$H]-thymidine was measured by liquid scintillation counting (1219 RackBeta, LKB Wallac, Turku, Finland) and proliferation was expressed as counts per minute (c.p.m.).

### *2.10. Cytokine determination by ELISA*

Cytokine concentration was measured in the medium conditioned by MLN cells ($1 \times 10^6$/well) which were cultured in 96-well plates for 48 h in medium solely (non-stimulated production) or in the presence of 1 μg/ml of ConA (stimulated production). Enzyme-linked immunosorbent assays (ELISA) for rat IFN-γ and for mouse IL-17 cross-reactive with rat IL-17 (eBioscience Inc., San Diego, CA, USA), and for rat IL-10 (R&D systems, Minneapolis, USA) were used according to the manufacturer's instructions. Cytokine titers were calculated by a reference to standard curve constructed using known amounts of respective set-provided recombinant cytokines.

### *2.11. Data display and statistical analysis*

Results were expressed as means ± standard deviation (S.D.). Statistical analysis was performed using STATISTICA 7.0 (StatSoft Inc., Tulsa, Oklahoma, USA). Statistical significance was defined by Mann-Whitney U test. *P* - values less than 0.05 were considered significant.

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## 3. Results

### *3.1. General considerations*

Rats were given warfarin in drinking water at 0.35 mg/l (Low Warfarin Dose, LWFD) and 3.5 mg/l (High Warfarin Dose, HWFD) (Table 1). According to the formula for calculation of Human Equivalent Dose, HED (Reagan-Shaw et al., 2008), the dose given to rats daily corresponded to HED of 0.005 mg/kg i.e. 0.30 mg/60 kg (for LWFD), or 0.046 mg/kg i.e. 2.7 mg/60 (for HWFD). The latter value is on a par with human exposure of 2-5 mg per average weight (60 kg) (Hirsh et al., 1998).

Intake of HWFD resulted in progressive increase in mortality of AO rats with no lethality in DA rats (Fig. 1A). Internal as well as external hemorrhage (Fig. 1B and C) was observed at HWFD only in AO rats. No lethal outcomes were noted at LWFD.

Because of detrimental effects of high warfarin dose on AO rats, the effect of low warfarin (WF) dose was examined further. Intake of this dose resulted in an increase in the mean PT values in both strains (Fig. 2), but with higher mean values in AO compared to DA rats ($P < 0.01$).

Drop in total peripheral blood leukocyte numbers was observed in AO rats solely ($6.4 \pm 1.6 \times 10^9$/l in WF-treated as compared to $8.0 \pm 1.0 \times 10^9$/l in control rats, $P < 0.05$), but with no changes in relative numbers of neutrophil leukocytes, lymphocytes or other leukocytes (not shown). No changes were seen in plasma levels of liver enzymes AST and ALT in either of strains following warfarin administration.

### *3.2. Histology of the intestine*

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In contrast to tall and thin villi and narrow lamina propria in control animals, lightly irregular villi with neutrophils in lamina propria were observed in the duodenum of warfarin-treated AO rats (Fig. 3). Mitotic activity was noted in glandular epithelium of these rats. Impact of warfarin on villous architecture was noted in DA rats as well, with mononuclear cell infiltration and rare neutrophils. Similar changes were noted in jejunum of both strains (not shown).

### *3.3. Effects of warfarin on intestinal oxidative activity in AO and DA rats*

Examination of basic parameters of oxidative activity including malondialdehyde (MDA) content, superoxide dismutase (SOD) and catalase (CAT) activity in homogenates of duodenum (Table 2), showed increase in MDA levels and CAT activity in warfarin-treated AO rats and a decrease of SOD activity in both strains.

### *3.4. Effects of warfarin on mesenteric lymph nodes (MLN) in AO and DA rats*

To see if differential intestinal reactivity to warfarin administration was in relation with gut-associated lymphoid tissue activity, basic characteristics of mesenteric lymph nodes were determined next.

No difference in MLN cell numbers was observed between warfarin-treated AO ($51.2 \pm 13.1 \times 10^6$) or DA rats ($53.3 \pm 19.7 \times 10^6$) as compared to $68.5 \pm 32.0 \times 10^6$ and $62.1 \pm 14.0 \times 10^6$ cells in respective strain controls. Warfarin consumption exerted no direct toxicity to MLN cells judging by trypan blue exclusion assay and by MTT reduction assay for cell's metabolic viability (Table 3). Although 48 h incubation of MLN cells in culture resulted in numerical drop ($P > 0.05$ compared to values obtained in freshly isolated cells) of MTT reduction capacity, no differences were noted between control and warfarin-treated groups of either of strains.

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When effects of warfarin consumption on non-stimulated MLN cell proliferation were evaluated (Fig. 4), lower levels were seen in warfarin-treated compared to control AO rats, while higher levels (*vs.* controls) were noted in DA rats which consumed warfarin. Stimulation with ConA resulted in an increase in proliferation in both strains, but the response was lower in WF-treated *vs.* control AO rats, and with no differences between groups of DA rats. Both non-stimulated as well as ConA-stimulated proliferation was lower in WF-treated AO compared to WF-treated DA rats ($P < 0.01$).

Measurements of IFN-γ production by MLN cells revealed drop of both spontaneous as well as ConA-stimulated cytokine production in AO (compared to controls), in contrast to DA rats, where no effect of warfarin intake was seen (Fig. 5A). No effects of warfarin on non-stimulated and ConA-stimulated MLN cell IL-17 production were seen (Fig. 5B).

To see if decrease of IFN-γ production might be ascribed to IL-10, known inhibitor of this cytokine (Couper et al., 2008), MLN production of IL10 was measured next (Fig. 6). Both non-stimulated and ConA-stimulated IL-10 was lower in warfarin-treated AO compared to controls, while no changes were observed in DA rats.

## 4. Discussion

Effects of oral warfarin intake on the intestinal immune responses in two rat strains were examined in the present paper showing, for the first time genetically-based variations in the toxicity of oral warfarin intake in the gut.

Differential susceptibility of rats to warfarin-induced bleeding is in line with the well- known variations of susceptibility of humans to this adverse effect of warfarin (Landefeld and Beyth, 1993). Lethal outcome at HWFD as well as more pronounced prolongation of prothrombin time

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at LWFD in AO compared to DA rats speaks about greater susceptibility of AO *vs.* DA rats to effects of warfarin related to hemostasis. Differences in the intestinal response to warfarin consumption between AO and DA rats show that there are genetically-based variations in other aspects of warfarin toxicity as well.

In line with data from patients on anticoagulant therapy (Yang et al., 2014), histological analysis of duodenum and jejunum demonstrated intestinal toxicity of warfarin both in AO and DA rats. Leukocyte infiltration in intestinal tissue might have accounted for drop in numbers of leukocytes in peripheral blood. It reflects tissue response to injury in order to manage it and regain intestinal homeostasis. In line with data which showed prooxidant potential of warfarin in tissue such is skin (Kataranovski et al., 2005) or its relevance for kidney injury (Ware et al., 2013), increases in MDA levels in intestinal tissue from warfarin-treated AO rats imply oxidative stress in this tissue as well. It might have resulted from neutrophil leukocytes which powerful effector activities are known to exert tissue injury (Kruger et al., 2015). Changes in the activity of both SOD and CAT in intestinal homogenates of warfarin-treated AO as compared to DA rats (in which only SOD was changed) along with mitotic activity in the intestine of warfarin-treated AO rats reflect more pronounced efforts of this strain to prevent/manage tissue injury. Intestinal epithelium is anatomical tissue border and one of the main components of intestinal homeostasis which might be compromised by injury (Mowat, 2003). Both intestinal anti-oxidative activity (in both strains) as well as mitotic epithelial cell activity in warfarin-treated AO rats contribute to protection of vulnerable intestine.

Suppression of proliferative capacity as well as IFN-γ and IL-10 production in AO rats could not be ascribed to warfarin cytotoxicity, as no changes in MLN cell viability were noted. These negative effects of warfarin administration in AO rats depict this lymph tissue as

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microenvironment which is prone to suppression of cell activity. Differential effects or lack of the effect on some aspects of MLN activity from WF-treated DA rats show that this strain reacts differently to warfarin. Increased levels of non-stimulated proliferation of MLN cells from these rats might have resulted from stimulation by antigenic products of damaged intestinal epithelium which reached lymph nodes by afferent lymph. However, lack of differences in ConA-induced proliferation between WF-treated and control DA rats, show lower responsiveness of WF group to additional stimulation. As a matter of fact, calculation of stimulation index (SI) as the ratio of ConA-stimulated and non-stimulated proliferation, revealed lower ($P < 0.05$) SI values in warfarin-treated ($36.1 \pm 17.3$) compared to control DA rats ($72.8 \pm 22.1$).

Being the border between intestinal and the rest of immune system, mesenteric lymph nodes prevent systemic immune priming and induction of potentially harmful immune responses (MacPherson and Smith, 2006). Suppression of proliferation as well as of production of proinflammatory cytokine IFN-γ in warfarin-treated AO rats underscores commitment to the suppression of inflammatory activities in MLN of these rats. Low responsiveness to additional stimulation of MLN cells' proliferation as well as lack of the effects on MLN cytokines in warfarin-treated DA rats also contribute to creation of the microenvironment which is non-permissive for immune priming. Whatever the strategy is used, local suppression and/or unresponsiveness are ultimately devoted to prevent systemic immune priming.

Inhibition of IL-10, mostly known as inhibitory cytokine (Couper et al., 2008), might be related to the fact that it can express stimulatory/proinflammatory activity as well (Conti et al., 2003; Groux et al., 1998; Lelievre et al., 1998) and that in certain conditions can stimulate IFN-γ production as shown in human endotoxemia (Lauw et al., 2000). Inhibition of IL-10 along with inhibition of IFN-γ in MLN cells of warfarin-treated AO rats might be envisaged as safety

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mechanism in inhibition of proinflammatory cytokine-induced responses. Lack of effects of warfarin administration to MLN cell's IL-17 response in both strains might be understood in view of data which showed that its inhibition could result in intestinal inflammation (Abraham and Cho, 2009; Ogawa et al., 2004) and in view of current knowledge of the crucial role of this cytokine in maintenance of immune-mediated intestinal homeostasis (Morrison et al., 2011; Shen et al., 2014).

In conclusion, oral intake of warfarin in AO and DA rats resulted not only in differences in the susceptibility to anticoagulant effect between these two strains, but in intestinal toxicity as well, which is novel finding. Oxidative injury of the intestine and subsequent engagement of both enzymes of anti-oxidative defense in AO rats suggest higher susceptibility of this strain to tissue toxicity of warfarin (Fig. 7). Both strains put efforts to prevent generation of potentially harmful immune responses in gut associated lymph nodes, but use different strategies.

**Conflict of interest statement**

The authors declare that there are no conflicts of interest.

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**Figure captions**

**Fig. 1**. Effect of Low Warfarin Dose (LWFD, i.e. 0.35 mg/l) and High Warfarin Dose (HWFD, i.e. 0.35 mg/l) intake. (A) Mortality of rats following warfarin intake. (B) Internal hemorrhage in AO rats as a consequence of HWFD consumption. (C) External hemorrhage in AO rats following HWFD intake. Data are presented as mean values ± S.D. from six animals per group. Significance at * $P < 0.05$ *vs.* control (0).

**Fig. 2**. Prothrombin (PT) time following warfarin intake. Data are presented as mean values ± S.D. from six animals per group. Significance at * $P < 0.05$ *vs.* control (0).

**Fig. 3**. Microscopic appearance of duodenum following warfarin intake. Irregular villous architecture in AO (left up) and DA rats (right up). Mitotic activity in glandular epithelium of AO rats (insert left). Microscopic appearance of duodenum in control animals (insert right). Infiltration of mononuclear cells and neutrophils (arrows) in lamina propria of AO rats (left down) and mainly mononuclear cells in DA rats (right down).

**Fig. 4**. MLN cell proliferative response following warfarin intake. Non-stimulated and Concanavalin A (ConA)-stimulated proliferation. Data are presented as mean values ± S.D. from six animals per group. Significance at * $P < 0.05$ and ** $P < 0.01$ *vs.* control (0).

**Fig. 5**. MLN cell proinflammatory cytokine response following warfarin intake. (A) Non-stimulated and ConA-stimulated IFN-γ production. (B) Non-stimulated and ConA-stimulated IL-

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17 production. Data are presented as mean values ± S.D. from six animals per group. Significance at ** $P < 0.01$ *vs.* control (0).

**Fig. 6**. IL-10 response in MLN following warfarin intake. Data are presented as mean values ± S.D. from six animals per group. Significance at ** $P < 0.01$ *vs.* control (0).

**Fig. 7**. Schematic summary of oral warfarin intake effects in Albino Oxford (AO) and Dark Agouti (DA) rats. Abbreviations: PT (Prothrombin Time), WBC (White Blood Cells), MDA (Malondialdehyde), CAT (Catalase), SOD (Superoxide dismutase), IFN-γ (Interferon-gamma), IL-17 (Interleukin-17), IL-10 (Interleukin-10).

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**Table 1** Daily warfarin intake in AO and DA rats.

| | AO Warfarin dose (mg/l) | | DA Warfarin dose (mg/l) | |
|---|---|---|---|---|
| | 0.35 | 3.5 | 0.35 | 3.5 |
| Body weight (mg) | 312.7 ± 39.5 | 298.4 ± 21.3 | 247.0 ± 14.1 | 237.8 ± 11.9 |
| Water consumption (ml) | 32.0 ± 5.1 (23.3 – 40.0) | 35.3 ± 5.2 (28.3 – 42.2) | 22.1 ± 2.6 (16.7 – 26.8) | 20.1 ± 3.0 (16.7 – 26.9) |
| Warfarin intake (mg/kg/day) | 0.034 ± 0.007 (0.021 – 0.047) | 0.319 ± 0.114 (0.161 – 0.441) | 0.033 ± 0.004 (0.022 – 0.041) | 0.306 ± 0.053 (0.257 – 0.433) |

Data are expressed as mean values ± S.D. from six animals per group.

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**Table 2**
Intestinal oxidative activity following warfarin intake in AO and DA rats.

| | AO | | DA | |
|---|---|---|---|---|
| | 0 | WF | 0 | WF |
| MDA (%) | 100.0 ± 14.1 | 128.9 ± 11.3* | 100.0 ± 7.5 | 94.9 ± 8.9 |
| CAT (%) | 100.0 ± 22.2 | 138.8 ± 28.6* | 100.7 ± 3.4 | 103.1 ± 3.5 |
| SOD (%) | 100.0 ± 17.4 | 79.9 ± 12.4* | 100.0 ± 25.9 | 64.0 ± 8.2** |

Data are expressed as relative changes, calculated as percentages of the values obtained in control (warfarin 0 mg/l) animals, which were considered as 100% and presented as mean values ± S.D. from six animals per group.
Significance at:
* $P < 0.05$ and ** $P < 0.01$ *vs.* controls (0).

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**Table 3**
Lack of toxicity of warfarin to mesentric lymph node (MLN) cells.

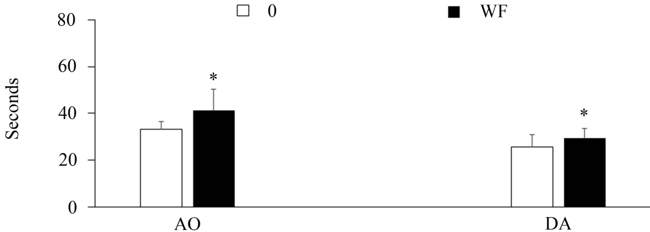
| | AO | | DA | |
|---|---|---|---|---|
| | 0 | WF | 0 | WF |
| Cell viability assay | | | | |
| Trypan blue exclusion (%) | 88.4 ± 4.0 | 87.6 ± 1.9 | 83.9 ± 6.7 | 81.3 ± 8.0 |
| MTT reduction ($A_{540nm}$) | | | | |
| freshly isolated cells | 0.287 ± 0.021 | 0.264 ± 0.038 | 0.240 ± 0.079 | 0.279 ± 0.072 |
| 48 h - cultured cells | 0.210 ± 0.091 | 0.211 ± 0.095 | 0.182 ± 0.071 | 0.231 ± 0.057 |

Data are expressed as mean values ± S.D. from six animals per group.

A

AO 0
AO LWFD
AO HWFD
DA 0
DA LWFD
DA HWFD

Survival (%)
100
80
60
40
20
0
*
0 5 10 15 20 25 30
Time (days)


0
WF
80
60
40
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Seconds
*
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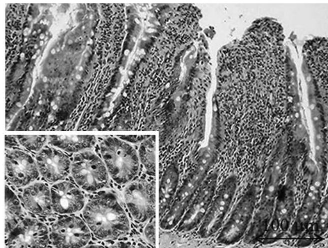
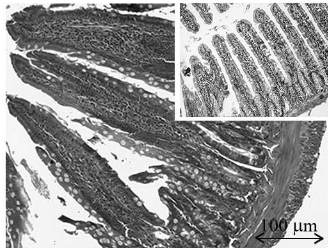
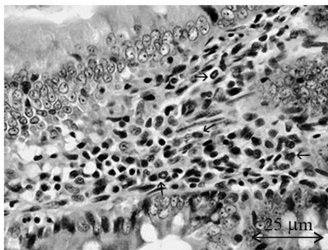
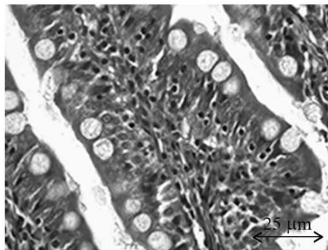
AO
DA

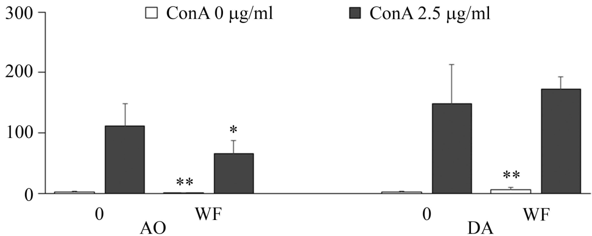
AO DA




ConA 0 μg/ml
ConA 2.5 μg/ml
300
200
100
0
3H proliferation (c.p.m. × 10^3)
*
**
**
0
WF
AO
0
WF
DA

A

ConA 0 μg/ml
ConA 1 μg/ml

IFN-γ (pg/ml)

8000
6000
4000
2000
0

**
**

0
WF
AO
0
WF
DA

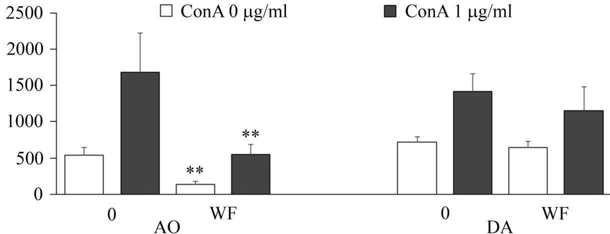
B

ConA 0 μg/ml
ConA 1 μg/ml

IL-17 (pg/ml)

8000
6000
4000
2000
0

0
WF
AO
0
WF
DA


ConA 0 μg/ml
ConA 1 μg/ml
IL-10 (pg/ml)
2500
2000
1500
1000
500
0
**
**
0
WF
AO
0
WF
DA

## EFFECTS OF SUBACUTE (30 days) ORAL WARFARIN INTAKE IN RATS

### GENERAL

| | |
|---|---|
| Lethal outcome | AO |
| Hemorrhage | AO |
| PT | Both strains, prolongation more pronounced in AO |
| Drop in WBC | AO |

### INTESTINAL EFFECTS

**Intestinal tissue**

| | |
|---|---|
| Histology | Damage, both strains, more severe in AO |
| Oxidative activity | |
| MDA | AO |
| CAT | AO |
| SOD | both strains |

**Draining lymph nodes**

| | |
|---|---|
| Cell proliferation | AO decrease<br>DA unchanged |
| Cytokine production | |
| IFN-γ | AO decrease<br>DA unchanged |
| IL-17 | unchanged, both strains |
| IL-10 | AO decrease<br>DA unchanged |