Environmental Toxicology and Pharmacology 41 (2016) 232–240

Contents lists available at ScienceDirect

Environmental Toxicology and Pharmacology

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

Transdermal toxicity of topically applied anticoagulant rodenticide warfarin in rats

Vesna Subota[a], Ivana Mirkov[b], Jelena Demenesku[b], Aleksandra Popov Aleksandrov[b], Marina Ninkov[b], Dina Mileusnic[b], Dragan Kataranovski[b,c], Milena Kataranovski[b,d,*]

[a] Institute for Medical Biochemistry, Military Medical Academy, Crnotravska 17, 11000 Belgrade, Serbia
[b] Department of Ecology, Institute for Biological Research "Sinisa Stankovic", University of Belgrade, Bulevar despota Stefana 142, 11000 Belgrade, Serbia
[c] Institute of Zoology, Faculty of Biology, University of Belgrade, Studentski trg 16, 11000 Belgrade, Serbia
[d] Institute of Physiology and Biochemistry, Faculty of Biology, University of Belgrade, Studentski trg 16, 11000 Belgrade, Serbia

ARTICLE INFO

Article history:
Received 20 August 2015
Received in revised form
11 December 2015
Accepted 14 December 2015
Available online 17 December 2015

Keywords:
Rats
Transdermal warfarin exposure
Peripheral blood leukocytes

ABSTRACT

Occupational/accidental exposure data have showed hemorrhage as a result of transdermal exposure to warfarin, however, other effects are not known. In the present study, the impact of epicutaneous application of 10 μg or 100 μg of warfarin (three times, once a day) on peripheral blood polymorphonuclear (PMN) and mononuclear cells (PBMC) was examined in rats. Both doses resulted in prolongation of prothrombin time and changes in hematologic parameters. Increases in PMN intracellular myeloperoxidase (MPO) activity were seen at higher warfarin dose and both doses resulted in higher percentages of granular $CD11b^+$ cells. In contrast, a decrease in PMN TNF and IL-6 production (ELISA) and gene expression (RT-PCR) was observed. Epicutaneous application of warfarin resulted in decreased numbers of PBMC, higher numbers of mononuclear $CD11b^+$ cells, but without effect on PMBC cytokine production. The data obtained showed differential effects of transdermal exposure to warfarin depending on leukocyte type and activity.

1. Introduction

Warfarin (4-OH coumarin) and its analogs are the principal vertebrate pesticides for controlling the commensal rodents. These anticoagulants are Vitamin K (VK) antagonist, and their rodenticide effect is based on the inhibition of the vitamin K-dependent (VKD) step in complete synthesis of a number of blood coagulation factors in liver including factor II (prothrombin, PT), factor VII (FVII), factor IX (FIX) and factor X (FX) required for normal blood coagulation (Shearer, 1990). Depletion of active form of these clotting factors leads to an increase in clotting time up to the point where no clotting occurs. Anticoagulants of the 4-hydroxicoumarin type have also been used in prophylactic medicine to prevent thromboembolic diseases in patients at risk for nearly 50 years (Furie, 2000).

Due to its important usage as a drug and a rodenticide, the underlying molecular mechanism of anticoagulant action of warfarin has been extensively studied. Warfarin and its analogs interfere with the cyclic interconversion of vitamin K and its 2,3 epoxide, by inhibiting vitamin K epoxide reductase (VKOR). Hidroquinone form of vitamin K (K1H2) is cofactor for γ-glutamil carboxylase, the vitamin K-dependent (VKD) enzyme which mediates posttranslational modification of glutamic (Glu) residues into γ-carboxyglutamic (Gla) acid residues, required for biological activity of VKD proteins (Furie, 2000). By inhibiting VKOR, warfarin affects the generation of biologically active VKD proteins not only involved in hemostasis, such as proteins relevant for regulation of bone metabolism (bone Gla protein, BGP and matrix Gla protein, MGP) (Price, 1988), cell growth and signaling (Nakano et al., 1997; Yanagita et al., 1999). Warfarin effect on BGP and MGP is responsible for developmental defects ("warfarin embryopathy") when consumed during pregnancy and for the loss of bone mass in patients on long-term warfarin therapy (WHO, 1995). Other adverse effects of warfarin therapy were observed and included intraperitoneal bleeding (Sagar et al., 2006), kidney injury (Brodsky et al., 2009, 2011), eosinophilia (Goudarzipour et al., 2015; Teragaki et al., 2012), sublingual hematoma (Bektas and Soyuncu, 2012) and intramural small bowel hematoma (Baser et al., 2013; Keskin et al., 2014). Warfarin also affects the processes not related to vitamin K metabolism including immune system reactions in humans. Early

* Corresponding author at: Department of Ecology, Institute for Biological Research "Sinisa Stankovic", University of Belgrade, Bulevar despota Stefana 142, 11000 Belgrade, Serbia. Tel.: +381 11 2078375; fax: +381 11 2761433.
E-mail address: milena@ibiss.bg.ac.rs (M. Kataranovski).

http://dx.doi.org/10.1016/j.etap.2015.12.006
1382-6689/

reports showed both immunostimulatory and immunosuppressive effects of warfarin in humans (Berkarda et al., 1983; Edwards and Rickles, 1978). Increased activity of natural killer (NK) cells in patients on warfarin therapy and antiproliferative effects of warfarin on some tumor cells (Bobek et al., 2005) created a basis for its use as an adjuvant antitumor therapy in humans (Bobek and Kovarik, 2004; Nakchbandi et al., 2006). Rodent studies also showed both suppression and stimulation of immune reactions by warfarin (Kataranovski et al., 2003; Perez et al., 1994) and kidney injury (Ware et al., 2013) and produced new data concerning warfarin-induced increase in blood pressure (Ware et al., 2015a). Thus, it is important to stay alert for (adverse) effects of this agent on cells and/or tissues.

Increased application of anticoagulant rodenticides in urban as well as in suburban/rural areas, has raised the concern about external exposure to these agents. In such settings, the direct contact with skin is an important factor of exposure both in persons exposed professionally (workers handling anticoagulant rodenticides while mixing and loading rodenticides, repairing and cleaning equipment) or in those who use easily available ready-to-use rodenticides in their households (WHO, 1995). The use of 0.5% warfarin solution for bait preparation during two weeks every few days (Fristedt and Sterner, 1965) and placing the baits containing 0.025% warfarin around the house once a week for an unspecified period of time (Abell et al., 1994) resulted in haemorrhagic diathesis. Collective exposure to warfarin was reported due to repeated use of talcum powders containing 1.7% to 6.5% of warfarin. Haemorrhagic disease was noted in 741 cases with 177 deaths (Martin-Bouyer et al., 1983). Cases of haemorrhage were reported among factory workers exposed to superwarfarins (brodifacoum and difenacoum) (Park et al., 1986) and bromadiolone and difethialone (Svendsen et al., 2002). Prompted by the latter case, the survey on the use of rodenticides (in kg), the use of protective equipment (mask and gloves) and hand hygiene was conducted for one month (Svendsen et al., 2002). Application of bromadiolone as powder (0. 15%) or meal (0.01%) was mean 50 kg (range 10–120 kg) and mean 1.2 kg (0–7 kg), respectively, while mean 23.7 kg (0–100 kg) of difethialone meal (0.0025%) was applied in one month (Svendsen et al., 2002). Gloves were used inconsistently (14% of workers) and hands were washed irregularly (33% workers after each rodenticide application and 67% before eating, after work). Beside 4-hydroxycoumarins (warfarin and superwarfarins), accidental dermal exposure to indandion anticoagulant rodenticides was reported (50 mL, 0.106% diphacinone) with resulting coagulopathy (Spiller et al., 2003). Occasional eye and skin irritation in persons handling some coumarin-based rodenticides was observed, which resulted in some precautionary statements concerning the hazard of skin contact with anticoagulant rodenticides and the necessity of immediate removal from the skin was stressed (WHO, 1995).

Inspired by these recommendations, the model of open epicutaneous application of warfarin in rats was introduced (Kataranovski et al., 2005). Using this model it has been shown that application of this agent to skin resulted in injury of epidermis (compact layer fissuring, keratohyaline granules, cell vacuolization in the granular layer, and supragranular exudation) and dermis (capillary congestion and capillary wall edema) (Popov et al., 2011). Skin responded to toxicity of warfarin by infiltration of $CD3^+$ cells (T cells), accumulation of mast cells and their activation/degranulation, as well as by stimulation of capillary endothelial cells and fibroblasts (Kataranovski et al., 2007; Zolotarevski et al., 2015) and production of proinflammatory cytokines by skin and epidermal cells (Popov Aleksandrov et al., 2015), depicting immune-potentiating activity of this agent in treated skin. However, a decrease in skin-draining lymph node responses to contact allergen dinitrochlorobenzene (DNCB) was seen as well (Kataranovski et al., 2003) showing immunosuppressive effects of warfarin. These and above cited data from human and rodent studies have shown that warfarin exerts variety of effects on immune cells. Differential effects of warfarin might rely on the differences in the type of cells and/or the activity that is examined.

Measurements of skin absorption of coumarin derivatives *in vivo* in rats (Yourick and Bronaugh, 1997), *in vitro* using diffusion cell skin absorption method (SAM) with rat, mouse and human skin (Beckley-Kartey et al., 1997) and with pig ear skin model (Jacques et al., 2010) showed that majority (~70%) of the applied dose was recovered from the skin with only 10–12% remaining in the skin itself. The initial data showed percutaneous absorption of warfarin in rats and peripheral blood polymorphonuclear cells' priming for respiratory burst as a result of transdermal exposure (Kataranovski et al., 2008). In order to examine further consequences of systemic absorption of warfarin, effects of epicutaneous application of this agent to blood hematology and peripheral blood leukocytes were examined in rats. Basic aspects of activity of both peripheral blood polymorphonuclear leukocytes (PMN)/granulocytes and mononuclear cells (PBMC) isolated from rats following repeated (one time daily, for three consecutive days) application of warfarin to skin were examined. Such a regimen of application was employed to mimic *in vivo* situation of professional exposure to this agent.

2. Materials and methods

2.1. Chemicals

Warfarin sodium (WF) (Serva Fein Biochemica, Heidelberg, Germany) was dissolved in endotoxin-free saline. Lypopolysaccharide (LPS), three-(4,5-dimethyl-thiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT), phorbol-12-myristate 13-acetate (PMA), *N*-(1-naphtyl) ethylenediamine dihydrochloride, sulfanilamide (*p*-aminobenzenesulfonoamide), hexadecyltrimethylammonium bromide (HTAB), *o*-dianisidine dihydrochloride, myeloperoxidase (MPO) and dextran T-500 were purchased from Sigma (Sigma Aldrich., St. Louis, MO, USA). LPS was dissolved in culture medium and MTT was dissolved in phosphate buffered saline (PBS, pH 7.2). Sodium nitrite was from Fluka Chemika (Buchs, Switzerland) and hydrogen peroxide (H_2O_2) from Zorka Farma (Sabac, Serbia). All solutions for cell culture experiments were either prepared under sterile conditions or were sterile filtered (Minisart, pore size 0.20 μm; Sartorius Stedim biotech, Goettingen, Germany) before use. Dextran T-500 as 6% solution was prepared in pyrogene-free saline and autoclaved (110 °C). Culture medium RPMI-1640 supplemented with 2 mM glutamine (PAA Laboratories, Pasching, Austria), 20 μg/mL gentamycine (Galenika a.d., Zemun, Serbia), 5% (v/v) heat inactivated fetal calf serum (PAA Laboratories, Pasching, Austria) was used in cell culture experiments. Monoclonal antibody OX-42 (mouse anti-rat CD11b/CD11c) and FITC-conjugated F(ab′)2 goat anti-mouse IgG were purchased from Serotec Ltd, Bicester, UK and eBioscience Inc., San Diego, CA, USA, respectively. Lysis buffer (eBioscience Inc., San Diego, CA, USA) was used for red blood cell lysis.

2.2. Animals and treatment

All animal procedures were complied with the Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes and approved by the Ethical Committee of the Institute for Biological Research "Sinisa Stankovic" (IBISS), University of Belgrade. Male Dark Agouti (DA) rats (IBISS), 10–12 weeks old, weighing 200 g were used. They were maintained at 12 h photoperiod, 21–24 °C temperature control and water and