



Oral warfarin intake affects skin inflammatory cytokine responses in rats

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ABSTRACT

Warfarin is an anticoagulant used in prevention/prophylaxis of thromboembolism. Besides the effects on coagulation, non-hemorrhagic reactions have also been documented. Although cutaneous reactions were reported in some patients, the impact on skin immunity was not explored. In the present paper, the effect of 30-day oral warfarin intake on skin cytokine responses in rats was analyzed. Increased release of inflammatory cytokines (TNF, IL-1 β and IL-10) was noted by skin explants from rats which received warfarin, but without effect on IL-6. No impact on epidermal cell cytokine secretion was seen, except a tendency of an increase of IL-6 response to stimulation with microbial product lipopolysaccharide (LPS). Topical application of contact allergen dinitrochlorobenzene (DNCB) resulted in slight (numerical solely) increase of TNF release by skin explants of warfarin-treated animals, while epidermal cells responded by increased secretion of all four cytokines examined. The data presented provide new information on the potential of oral warfarin to modulate skin innate immune activity.

1. Introduction

Although in use for more than 60 years, warfarin is still the most widely used anticoagulant drug in the world (Pirmohamed, 2006). Its use (according to prescriptions data) increased by 45% from 1998 to 2004 (Aguilar and Hart, 2005). Warfarin affects interconversion of Vitamin K (VK) and its 2,3 epoxide by inhibiting vitamin K epoxide reductase (VKOR). As a consequence of VKOR inhibition, depletion of hydroquinone form of VK (K1H2) (a cofactor of γ -glutamyl carboxylase which enables carboxylation of glutamyl (Gla) residues on precursors of several proteins involved in hemostasis) occurs and undercarboxylated, biologically non-active blood coagulation factors are generated including factor II (prothrombin, PT), factor VII (FVII), factor IX (FIX) and factor X (FX) (Furie, 2000). Because of narrow therapeutic index, it is difficult to maintain defined range of anticoagulation in patients and there is a risk of bleeding (due to warfarin overdose) or thrombotic events (at too low warfarin dose) (Boulanger et al., 2006).

According to data, in Great Britain since 2004, warfarin has reached the third position on the list of drugs resulting in admission to hospital because of its adverse effects (Pirmohamed et al., 2004). The major undesired effect of warfarin is bleeding (Linkins et al., 2003). Major

bleeding is the most important clinical manifestation of oral warfarin therapy which affects a variety of tissues and organs (Linkins et al., 2003) including central nervous system (Flaherty et al., 2007). Non-hemorrhagic adverse effects of warfarin were also reported and skin was recognized as one of the affected tissues (Gallerani et al., 1995).

Warfarin-induced skin necrosis (WISN) is a rare (0.01%–0.1%) clinical complication in patients on oral warfarin therapy (Chan et al., 2000). Cutaneous hemorrhage with vascular damage and thrombosis with skin necrosis is the typical histological feature in these patients (Ad El et al., 2000). Signs of immune activity (proinflammatory cytokine TNF in necrotic tissue and endothelial adhesion molecule at the periphery of the lesion) were described in some patients (Hermes et al., 1997). Deficiency/low levels of proteins C and S, as well as anti-thrombin III deficiency are considered as major risk factors for WISN (Nazarian et al., 2009). Besides WISN, other adverse health effects of oral warfarin therapy were reported including hypersensitivity reactions such as ecchymoses and purpura, skin eruptions (vesicular and maculopapular), purple toes and exanthematous reactions (Bircher et al., 2006; Kakagia et al., 2014; Nazarian et al., 2009; Panunzialman and Falanga, 2010). Hypersensitivity to different coumarins was also described (Kruis-de Vries et al., 1989) and positive response to re-

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challenge was noted (Spyropoulos et al., 2003). Warfarin-induced leukocytoclastic vasculitis (LCV) was rarely reported and only several cases were described (Howitt et al., 1982; Hsu et al., 2012; Jiménez-Gonzalo et al., 1999; Susano et al., 1993; Tamir et al., 1994; Tanay et al., 1982; Yaghoubian et al., 2005). It could be associated with skin necrosis or as a separate entity (Kurt et al., 2007). Most common histological finding of involved skin are structural changes of dermal vasculature (sometimes with fibrinoid necrosis) and perivascular infiltration of polymorphonuclear/neutrophil and mononuclear cells. Oral warfarin also causes systemic hypersensitive reactions characterized by eosinophilia (Goudarzipour et al., 2015; Hall and Link, 1981; Jo et al., 2011; Kuwahara et al., 1995; Teragaki et al., 2012) and presence of eosinophils was noted in the skin and kidneys in some patients (Kapoor and Bekaii-Saab, 2008). All these studies showed that cutaneous effects of oral warfarin therapy had involved immune system cells, but there were no data concerning effects on components of regional skin immune system in such settings.

Using a model of repeated epicutaneous administration of warfarin in rats (Kataranovski et al., 2007, 2003; Popov Aleksandrov et al., 2015) it has been shown that skin is a target organ for immunomodulatory effects of this agent. Influence of 30-days oral warfarin intake on basic aspects of peripheral blood leukocyte activity in rats (Belij et al., 2012; Popov et al., 2013) at doses which resulted in visceral organ hemorrhage (Mirkov et al., 2016) depicted immunomodulatory effects of this agent when received as oral treatment.

In view of these and above cited data, the aim of the present paper was to examine whether oral warfarin intake exerted influence on immune/inflammatory activity in the skin. Therefore, the effect of 30-day oral warfarin intake on the skin and epidermal cell inflammatory cytokine (TNF, IL-1 β and IL-6) response was examined in rats. Because IL-10 is involved in the inflammatory responses of keratinocytes, this cytokine was measured too. The impact of warfarin on responsiveness to external stimulation by skin sensitizer dinitrochlorobenzene (DNCB) was examined as well. Data obtained showed stimulatory effects of warfarin on skin inflammatory cytokine responses, which is a novel finding.

2. Materials and methods

2.1. Chemicals

Warfarin sodium was purchased from Serva Fein Biochemica (Heidelberg, Germany). Dispace II was obtained from Boehringer (Manheim, Germany) and trypsin solution from Difco (Lawrence, KS, USA). Hexadecyltrimethylammonium bromide (HTAB), *o*-dianisidine dihydrochloride, myeloperoxidase (MPO), three-(4,5-dimethyl-thiazol-2-yl)-2,5 diphenyl-tetrazolium bromide (MTT), lypopolysaccharide (LPS) and phenylmethanesulfonyl fluoride (PMSF) were all purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA). 1-Chloro-2,4-dinitrochlorobenzene (DNCB) was obtained from BDH Chemicals Ltd., London, UK and dissolved in 4:1 acetone:olive oil (vehicle). Hydrogen peroxide (H₂O₂) was purchased from Zorka Farma (Sabac, Serbia). Dispace II was dissolved in culture medium. All solutions for cell culture experiments were either prepared under sterile conditions or were sterile filtered (Flowpore, pore size 0.22 μ m) before use. Culture medium RPMI-1640 (Biowest, Nuaille, France) supplemented with 2 mM glutamine, 20 μ g/ml gentamycin (Galenika a.d., Serbia), 5% (v/v) heat inactivated fetal calf serum (Biowest, Nuaille, France) was used in cell culture experiments.

2.2. Animals

All procedures used herein complied with the European Communities Council Directive (86/609/EEC) and were approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research “Sinisa Stanković” (IBISS), Belgrade, Serbia.

Male Dark Agouti (DA) rats eight to ten weeks old, conventionally housed at the IBISS animal facility were used. Eight animals were assigned to each treatment group. All analyses were done in samples of eight animals.

2.3. Warfarin treatment

Warfarin sodium was prepared in drinking water at concentration of 3.5 mg/l and was given to rats for 30 days. Warfarin was replaced with freshly prepared solution twice a week. Control animals received water solely. The effectiveness of warfarin intake was checked by measurements of prothrombin time (PT) and partial thromboplastin time (PTT) (described below), which are the biological markers of the anticoagulant effect of warfarin.

2.4. Prothrombin time (PT) and partial thromboplastin time (PTT)

Prothrombin time (PT) and partial thromboplastin time (PTT) were determined in blood samples obtained from abdominal artery and diluted with 3.8% sodium citrate (final blood to citrate ratio of 5:1). PT was determined by one-stage method using citrate plasma and Thromborel S reagents (Behring Diagnostics GmbH, Marburg, Germany) with Siemens equipment. The kaolin-activated PTT was determined by one stage method using Pathrombin (Behring).

2.5. Histology

Skin samples were taken 24 h following completion of warfarin intake. They were fixed in 4% buffered formaldehyde (pH 6.9) and embedded in paraffin wax for sectioning at 5 μ m. Hematoxylin and eosin (H & E)-stained histology slides were subsequently analyzed by a certified histopathologist in a blinded manner using a Coolscope digital light microscope (Nikon Co, Tokyo, Japan).

2.6. Skin explants culture

Skin was excised 24 h following completion of warfarin intake, cleared of subcutaneous tissue, cut into pieces (~10 $\times$ 10 mm) and placed in duplicate of 0.4 ml complete culture medium. After 48 h culture supernatant (conditioned medium) was collected by centrifugation.

2.7. Epidermal cell preparation and culture

Skin was cut into small pieces and incubated in dispace (2.5 mg/ml) at 4 °C overnight, in order to separate the epidermis from dermis. Epidermal sheets were then put into trypsin-glucose solution (0.25% trypsin; 0.1% glucose) for 30 min at 37 °C, and single epidermal cell suspension was prepared. Cells were counted by improved Neubauer hemocytometer, and then placed at 5 $\times$ 10⁵ cells/ml in wells of 96-well plates in culture medium and cultured for 48 h. To examine the effect of warfarin on stimulated epidermal cell cytokine responses, cells were cultured with 100 ng/ml of lypopolysaccharide (LPS).

2.8. MTT assay for cell viability

Epidermal cells viability was determined by a quantitative colorimetric assay described for adherent cells (Oez et al., 1990) which is based on metabolic reduction of tetrazolium salt MTT to a colored end product, formazan. Cells were added to wells of a 96-well plate (1 $\times$ 10⁵ epidermal cells/well) and incubated with 500 μ g/ml of MTT for 3 h. Formazan produced by the cells was dissolved by overnight incubation in 10% sodium dodecyl sulfate (SDS) – 0.01N HCl and absorbance of extracted chromogen was then measured at 540/650 nm by ELISA 96-well plate reader (GRD, Rome, Italy).

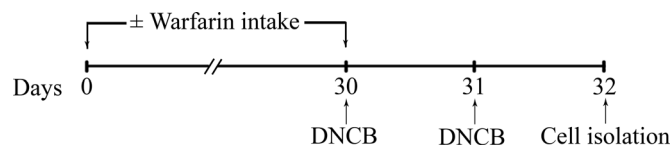


Fig. 1. Schematic presentation of experimental design.

2.9. Cytokine determination by ELISA

Tumor necrosis factor (TNF), interleukin -1 β , -6 and -10 (IL-1 β , IL-6 and IL-10) concentrations in medium conditioned by skin explants or epidermal cells were evaluated using enzyme-linked immunosorbent assays (ELISA) for rat TNF (eBioscience Inc., San Diego, CA, USA), rat IL-1 β , rat IL-6 and rat IL-10 (R & D systems, Minneapolis, MN, USA). Cytokines titer was calculated by the reference to the standard curve constructed with known amounts of the recombinant cytokine.

2.10. Contact sensitization with DNCB

On day 30 of warfarin or water intake, 100 μ l of 0.4% (w/v) DNCB in vehicle (acetone/olive oil, 4:1) was applied for 2 consecutive days to the upper part (approximately 16 cm²) of dorsum, thoroughly clipped out of the fur. Histological analysis, skin explant and epidermal cell cytokine responses were examined 24 h following the last DNCB administration (Fig. 1).

2.11. Determination of myeloperoxidase (MPO) activity in the skin

Skin was excised 24 h following the last DNCB administration, weighed and 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 TrisHCl pH 7.6, 1 mM EDTA, 250 mM sucrose) containing 1 mM phenylmethanesulfonyl fluoride (PMSF), on ice. MPO activity was measured in supernatants obtained by centrifugation at $1000 \times g$ for 15 min, at 4°C and assessed on the basis of the oxidation of *o*-dianisidine dihydrochloride by cells (Bozeman et al., 1990). Enzyme activity was evaluated by the addition of 33 μ l of skin homogenate to 966 μ l of substrate solution (0.167 mg/ml *o*-dianisidine dihydrochloride and 0.0005% H₂O₂ in 50 mM potassium phosphate buffer, pH 6.0). Absorbance was read at 450 nm at 3 min intervals during 10 min against the standard of MPO. Values were expressed as MPO per gram of wet tissue.

2.12. Data display and statistical analysis

Results were expressed as means $\pm$ standard deviation (Tables) or the scatter plots with means (Figures). Statistical analysis was performed by 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.

3. Results

3.1. General considerations

Rats received 0.0875 mg of warfarin (the value derived from warfarin concentration in water, i.e. 3.5 mg/l and the average water volume consumed per rat i.e. 25 ml/d) each day, which equates to the dose of 0.4 mg/kg (average rat body mass of 215 g). According to the formula for calculation of Human Equivalent Dose, HED (Reagan-Shaw et al., 2008), the HED (in mg/kg) is equal to the animal dose (mg/kg) multiplied by the [Animal K_m (i.e. 6)/the Human K_m (i.e. 37)]. Daily intake of 0.4 mg/kg warfarin by rats is adequate to HED of 0.06 mg/kg, a value corresponding to the level of 3.6 mg/60 kg which is in the range

Table 1

Prothrombin and partial thromboplastin time following warfarin intake.

	Warfarin intake	
	(–)	(+)
Prothrombin time (s)	24.5 $\pm$ 7.2	57.3 $\pm$ 12.8*
Partial thromboplastin time (s)	55.1 $\pm$ 13.3	199.9 $\pm$ 41.8*

Data are expressed as mean values from eight animals $\pm$ S.D.

Significance at: **P* < 0.05 vs. control (warfarin (–)).

of 2–5 mg per average weighed person that, according to literature reports, have been indicated as warfarin dose for humans (Hirsh et al., 1998). Such warfarin dose resulted in increases in the mean prothrombin time and in partial thromboplastin time (Table 1) confirming the access of warfarin to general circulation.

3.2. Histological analysis

Histological analysis of the skin (Fig. 2) revealed slightly increased cellularity of the dermis and dilated hair follicles in group of rats which received warfarin. No changes were seen in subcutaneous tissue (Supplement 1).

3.3. Inflammatory cytokine release from skin explants and epidermal cells

Measurements of inflammatory cytokine levels (Fig. 3, Panel A) showed higher levels of TNF and IL-1 β in medium conditioned by skin explants from rats which received warfarin compared to rats which received water solely. No effect was seen on IL-6 release. Warfarin intake exerted no influence on inflammatory cytokine secretion by epidermal cells (Fig. 3, Panel B).

To see if warfarin affects viability of epidermal cells (which could affect their cytokine secretion potential) the capacity of reduction of MTT salt was measured next (Table 2). No differences were noted in the MTT assay between epidermal cells isolated from the skin of rats which received warfarin or water. There were no changes in epidermal cells viability following 48 h in culture as well.

To examine whether warfarin intake affects cell's responsiveness to inflammatory stimuli, secretion of cytokines by epidermal cells cultured in the presence of LPS was examined next (Table 3). No differences were noted between TNF and IL-1 secretion by cells from controls and warfarin-exposed rats, while increase of IL-6 was observed in warfarin-exposed group.

3.4. Effect of warfarin on skin and epidermal cell inflammatory cytokine responses to external stimulation in vivo

To see if warfarin intake could modulate skin responses to external stimulation, contact allergen DNCB was applied to the skin following the completion of anticoagulant exposure. Histological analysis (Fig. 4) showed discrete, nonspecific reactive changes in the skin barrier, reactivity of mesenchymal cells, dermal edema and scarce cell infiltrates in skin of control rats group. More dilated follicles (dilatation often between hair and epithelium) were noted as well. Similar changes were observed in DNCB-treated skin of rats which received warfarin, but in this group follicle degeneration was the most prominent feature. No changes were observed in subcutaneous tissue (Supplement 1).

Measurements of MPO in homogenates of the skin of these groups showed similar levels of enzyme activity (0.075 $\pm$ 0.005 U/g in control compared to 0.066 $\pm$ 0.014 U/g in group of rats which received warfarin).

Measurements of cytokine levels in medium conditioned by skin from rats which received warfarin and were sensitized with DNCB thereafter (Fig. 5, Panel A) showed lack of warfarin effect on these

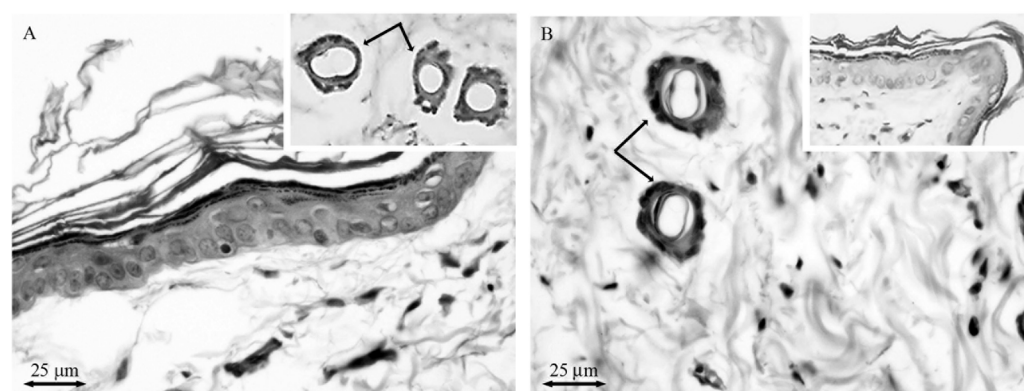


Fig. 2. Histological appearance of skin: (A) Control group. Hair follicles (insert). (B) Warfarin group. Epidermis (insert). Arrows indicate hair follicles which are dilated in warfarin-treated group.

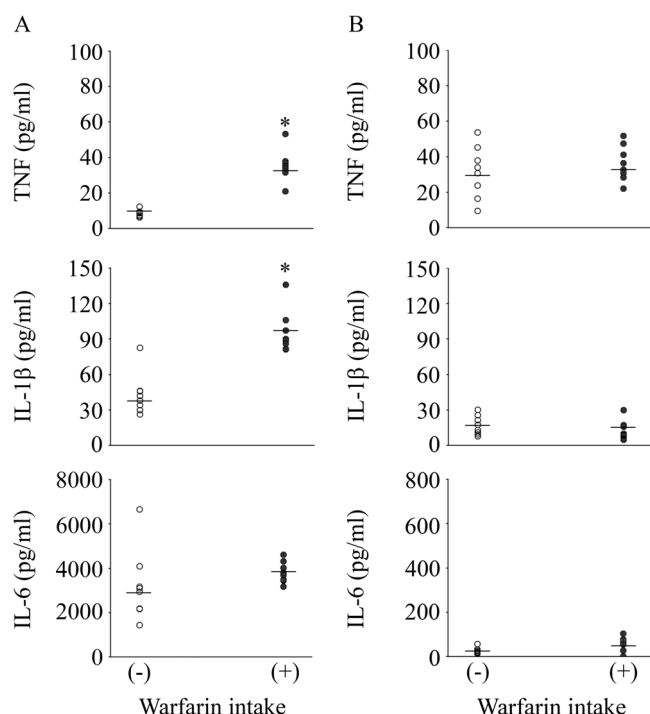


Fig. 3. Inflammatory cytokine secretion from skin explants (Panel A) or epidermal cells (Panel B). Dots represent individual values from eight animals and the mean value has been indicated. Significance at: * $P < 0.05$ vs. controls.

Table 2
MTT reduction by epidermal cells.

MTT reduction (A_{540})	Warfarin intake	
	(–)	(+)
Freshly isolated cells	0.17 ± 0.03	0.16 ± 0.05
48 h – cultured cells	0.15 ± 0.02	0.16 ± 0.01

Data are expressed as mean values from eight animals $\pm$ S.D.

Table 3
Effect of warfarin on LPS-stimulated cytokine production by epidermal cells.

	Warfarin intake	
	(–)	(+)
TNF (pg/ml)	29.0 ± 10.2	26.6 ± 8.1
IL-1 β (pg/ml)	25.1 ± 5.6	23.3 ± 5.7
IL-6 (pg/ml)	149.7 ± 66.6	$277.6 \pm 120.3^*$

Data are expressed as mean values from eight animals $\pm$ S.D.

Significance at: * $P < 0.05$ vs. controls.

inflammatory mediators (except for TNF, where owing to great variations, only numerical, $P = 0.12$, increase was seen). In contrast, significant impact of warfarin was seen on epidermal cells' cytokine responses to topical DNCB (Fig. 5, Panel B).

3.5. Effect of warfarin on skin and epidermal cell anti-inflammatory cytokine responses

Measurements of anti-inflammatory cytokine (IL-10) levels (Fig. 6, Panel A, up) showed higher levels of cytokine in the medium conditioned by skin explants from rats which received warfarin compared to rats which received water solely. Warfarin intake however, exerted no influence on IL-10 secretion by epidermal cells (Fig. 6, Panel A, down). No differences were noted between cytokine produced following stimulation with LPS (37.4 ± 19.2 pg/ml and 51.0 ± 28.4 pg/ml, cells from controls and warfarin-exposed rats, respectively). Measurements of IL-10 concentrations in medium conditioned by the skin from rats which received warfarin and were subsequently sensitized with DNCB (Fig. 6, Panel B, up) showed lack of warfarin effect on this cytokine. In contrast, significant impact of warfarin was seen on epidermal cells' cytokine production in response to topical DNCB (Fig. 6, Panel B, down).

4. Discussion

In the present paper, the effect of oral warfarin intake on the skin of rats was examined by analyzing skin and epidermal cell inflammatory cytokine secretion. Warfarin intake resulted in increased release of inflammatory cytokines (TNF and IL-1 β) and IL-10 from skin, but no effect on epidermal cells was seen. Exposure to contact allergen DNCB resulted in increased secretion of TNF, IL-1 β , IL-6 and IL-10 from epidermal cells of rats which received warfarin before topical exposure sensitization. These data provide new information on the potential of oral warfarin to prime skin for innate immune activity.

Follicle dilatation noted in groups which received warfarin (which was more pronounced and associated with follicle degeneration following topical treatment of these rats with DNCB) depicts subtle cutaneous effects of this anticoagulant. Increases in inflammatory cytokine secretion in these groups might be related to the observed change, as hair follicle is the area of immune privilege the break of which is crucial for alopecia areata (Christoph et al., 2000). In this regard, hair loss was reported to be associated with the use of warfarin in some patients (Watras et al., 2016).

Increases in TNF and IL-1 β cytokine release from skin explants of rats which received warfarin have shown potential of this therapeutic to stimulate inflammatory cytokines in the skin, but the lack of the effect on IL-6 suggested some selectivity in this effect. Presence of TNF in patients who developed skin necrosis after the start of oral coumarin therapy (Hermes et al., 1997) suggest the impact of warfarin on skin cytokine responses as well. In corroboration, TNF (and IL-1 β) release from skin

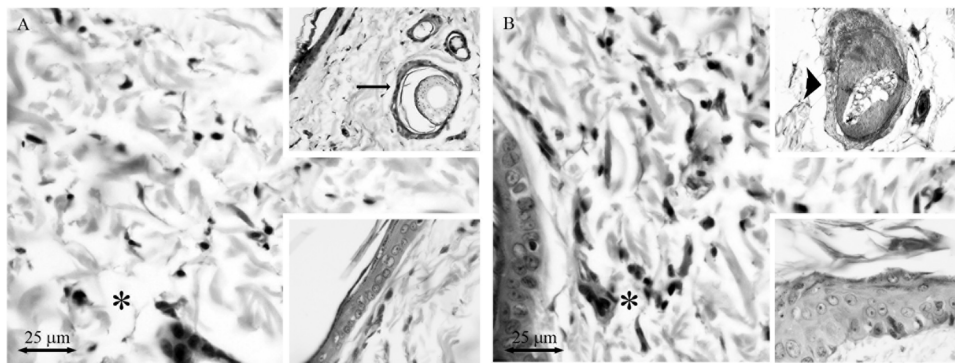


Fig. 4. Histological appearance of skin following topical application of DNCB: (A) Control group. Thinned epidermal granular layer (lower insert). Dilated hair follicle (arrow, upper insert). Tissue edema (asterisk). (B) Warfarin group. Widened epidermis with irregular granular layer (lower insert). Degenerated hair follicle (arrowhead, upper insert). Tissue edema (asterisk).

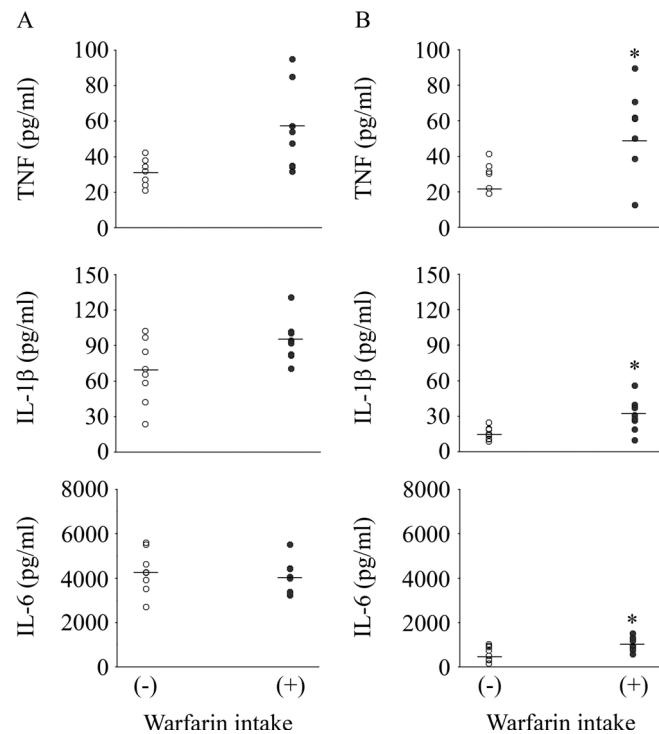


Fig. 5. Inflammatory cytokine secretion following topical application of DNCB. Skin explants (Panel A). Epidermal cells (Panel B). Dots represent individual values from eight animals and the mean value has been indicated. Significance at: * $P < 0.05$ vs. controls.

explants of rats exposed epicutaneously to warfarin was noted previously by our group (Popov Aleksandrov et al., 2015). Oral warfarin intake exerted influence on IL-10 skin response as well. Increased levels of this cytokine are in line with data which showed its stimulatory effects in several models and experimental systems including the process of leukocyte migration and leukocyte and endothelial cell activation (Byrne and Reen, 2002; Huang et al., 2001; Jinquan et al., 1993; Vora et al., 1996). As a matter of fact, expression of IL-10 is concordant with other innate mediators with potent proinflammatory properties early in the response, and only later anti-inflammatory/immunoregulatory effects of this cytokine is expressed (Mocellin et al., 2003). Lack of the effect of oral warfarin intake on epidermal cell secretion of TNF, IL-1 β and IL-10 was not caused by cytotoxicity, as no effects on viability of freshly isolated or 48 h cultured epidermal cells were shown. It rather suggests contribution of other cells from the skin to increased cytokine release by skin explants. In this regard, fibroblasts, endothelial cells and mast cells are skin cells which have capacity to produce TNF (Bashir et al., 2009; Ranta et al., 1999; Suto et al., 2006) while fibroblasts (Boxman et al., 1993) and Langerhans cells (Enk et al., 1993) are reported as sources of IL-1. Dermal dendritic cells/macrophages might be responsible for increase of IL-10 as these leukocyte types are the known sources of cytokine (Byrne and

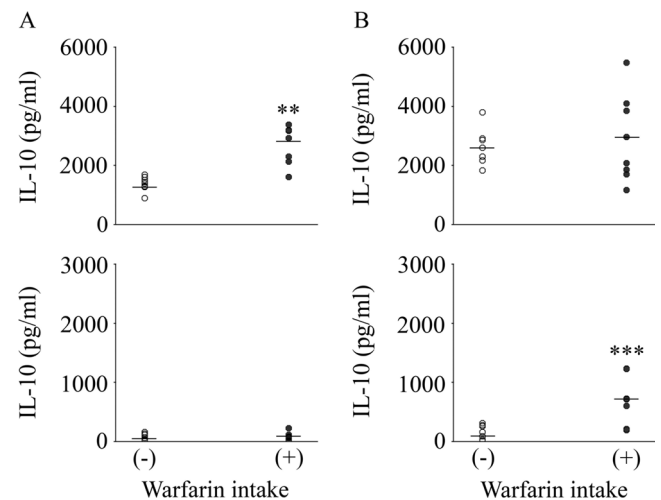


Fig. 6. Skin and epidermal cell IL-10 secretion. Effect of warfarin on IL-10 production (Panel A). IL-10 production following topical application of DNCB (Panel B). Skin explants (up). Epidermal cells (down). Dots represent individual values from eight animals and mean value is indicated. Significance at: ** $P < 0.01$ and *** $P < 0.001$ vs. controls.

Reen, 2002; Huang et al., 2001). Lack of the effect of LPS on epidermal cell production of TNF, IL-1 and IL-10 implies that cells of this skin layer are unresponsive to exogenous stimuli, though an increase of IL-6 secretion implies that absence of response seems not to be valid for this cytokine. However, increases in TNF, IL-1 β , IL-6 and IL-10 secretion by epidermal cells isolated from the skin sensitized with DNCB showed that warfarin might prime these cells for responses to some stimuli. Lack of the effect of warfarin intake on skin explant cytokine responses to DNCB might be perceived in the light of a well-known fact that among skin cellular components, epidermal cells are chiefly responsible for the contact sensitizers-induced inflammation in this tissue (Wilmer et al., 1994). However, inhibitory effect of warfarin could not be totally excluded, as this agent is capable of exerting both stimulatory as well as suppressive effects (Djokic et al., 2013; Eichbaum et al., 1979; Kurohara et al., 2008).

In conclusion, the data presented provide new information concerning the potential of oral warfarin intake to modulate skin innate immune activity. Oral warfarin intake resulted in stimulation of skin inflammatory cytokine (TNF, IL-1 β and IL-6) as well as IL-10 responses and primed epidermal cells responsiveness to external stimulation. Priming effect of warfarin on these cytokines, might, at least in some circumstances, increase skin susceptibility to adverse reactions.

Conflict of interest statement

The authors declare that there are no conflicts of interest in this study. The authors alone are responsible for the content of this manuscript.

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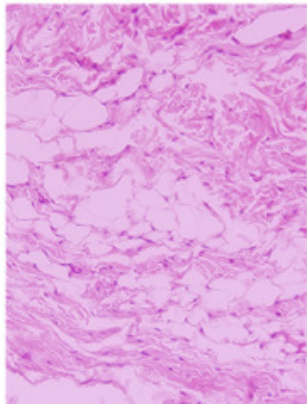
Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.etap.2017.06.027>.

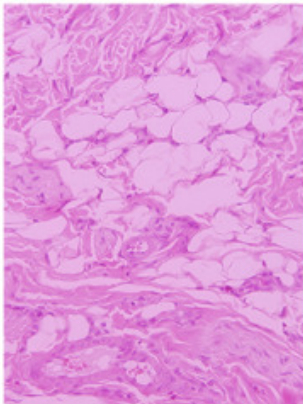
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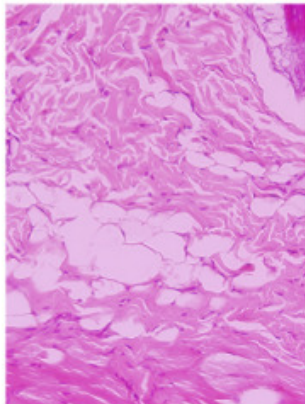
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