Original Article

Local Proinflammatory Effects of Repeated Skin Exposure to Warfarin, An Anticoagulant Rodenticide in Rats*

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Abstract

Objective: To evaluate the effects of epicutaneous application of anticoagulant warfarin, by examining the presence of tissue injury and immune/inflammatory activity in exposed skin.

Methods: Rats were exposed to warfarin by applying 10 μg of warfarin-sodium to 10-12 cm^2 skin (range 0.8-1 μg per 1 cm^2) for 3 consecutive days. Tissue injury was evaluated by lipid peroxidation, histomorphological changes and signs of reparative activity in skin. T cell infiltration and selected aspects of epidermal cell activity were examined as indicators of immune/inflammatory skin response to warfarin application.

Results: Repeated warfarin application exerted no effect on skin metabolic viability, but resulted in tissue injury (increased malondialdehyde, MDA, production, evident histo-morphological changes in epidermis and dermis depicting cell injury and death). Increased numbers of proliferating cell nuclear antigen ($PCNA^+$) cells indicated reparative processes in injured skin. Infiltration of $CD3^+$ cells (T lymphocytes) along with the increased production of tumor necrosis factor-α (TNF-α) by epidermal cells from warfarin-treated skin and their co-stimulatory effect in an *in vitro* T-cell activation assay demonstrated immunomodulatory effects of epicutaneous warfarin.

Conclusion: Presented data have documented tissue damage associated with immune/ inflammatory activity in skin exposed to warfarin. Observed effects are relevant to immunotoxic potential of this anticoagulant in settings of external exposure.

Key words: Rats; Warfarin; Epicutaneous exposure; Tissue damage; Skin inflammation

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INTRODUCTION

Warfarin (4-OH coumarin) and its analogs are the most effective agents used in rodent control. This group of anticoagulant is Vitamin K antagonist, and its 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 the liver, including Factor II (prothrombin, PT), Factor VII (FVII), Factor IX (FIX), and Factor X (FX) required for normal blood coagulation[1]. Depletion of active forms of these clotting factors leads to an increase in clotting time up to the point where no clotting

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occurs. Anticoagulants of the 4-hydroxycoumarin type have also been used to prevent thromboembolic disorders in patients at risk for nearly 50 years[2]. 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 a cofactor for γ-glutamil carboxylase, Vitamin K-dependent (VKD) enzyme which mediates posttranslational modification of glutamic (Glu) residues into γ-carboxyglutamic (Gla) acid residues, needed for the biological activity of VKD proteins[2]. By inhibiting VKOR, warfarin affects the generation of biologically active VKD proteins not involved in hemostasis, including proteins relevant to regulation of bone metabolism (bone Gla protein, BGP and matrix Gla protein, MGP)[3], cell growth[4-5] and signaling[6-8]. The effects of warfarin on BGP and MGP lie in growth and development ("warfarin embryopathy") when warfarin is consumed during pregnancy[9] and also in loss of bone mass in patients under long-term warfarin therapy[10-11]. It is thus important to stay alert to adverse effects of this agent on cell and tissue physiology.

Increased application of anticoagulant rodenticides in urban as well as in suburban/rural areas, has raised concerns over external exposure to these agents. In such settings, direct contact with skin is an important way of exposure either among persons exposed professionally (workers handling anticoagulant rodenticides while mixing and loading rodenticides, repairing and cleaning equipment) or among those who use easily available ready-to-use rodenticides in their households[12]. Reported incidents of warfarin intoxication from percutaneous absorption[13-17] indicate skin as an important way of entry of this chemical. Studies of percutaneous absorption of second generation hydroxycoumarin, flocoumafen, revealed that around 12% of the applied dose remained in the skin of rats for up to seven days[18]. Studies using the skin absorption model system *in vitro* demonstrated that topically applied coumarin is rapidly and extensively absorbed by human, rat, and mouse skin, remaining there for 72 h following application[19]. These data implicate that skin serves as a place where these agents might linger and exert their effects.

Skin is not just a passive interface between the host and the hostile environment, but functions as a toxicological, biochemical and immunological barrier[20] providing thus protection for the host. Our previous studies showed changes in skin after single application of warfarin to skin of experimental rats[21-22], suggesting proinflammatory potential of this anticoagulant. As the inflammatory activity of many environmental insults is linked with their tissue damaging properties[23-26], the aim of this study is to examine whether warfarin application is associated with tissue injury and inflammation. Repeated (one time daily, for three consecutive days) application of warfarin at a dose of 10 μg/10-12 cm^2 to skin, which had been previously shown to result in mild changes in rat skin following single application, was employed. Selected aspects of skin changes (lipid peroxidation, resident skin cell appearance and signs of reparative activity) were regarded as signs of tissue injury in skin exposed to warfarin. Examination of the immune/inflammatory activity in warfarin-treated skin, including evaluation of T lymphocyte skin infiltration and activity of epidermal cells isolated from exposed skin, was conducted in parallel. Data were obtained, which demonstrated that epicutaneous warfarin application resulted in tissue injury and that this was associated with immune/inflammatory activity in treated skin.

MATERIALS AND METHODS

Chemicals and Monoclonal Antibodies (mAb)

Warfarin sodium (WF) (Serva, Germany) was dissolved in endotoxin-free saline. Dispase II (Boehringer, Mannheim, Germany) was dissolved in liquid culture medium RPMI-1640 and trypsin (Difco, Cansas, USA) was dissolved in phosphate buffered saline (pH 7.2) at 0.25% with 0.1% glucose added. Three-(4,5-dimethyl-thiazol-2yl)-2,5-diphenyl-tetrazo lium bromide (MTT), Concanavalin A (ConA), 2-thiobarbituric acid, malondialdehyde bis (dimethyl acetal) (MDA) and trichloroacetic acid were purchased from Sigma (Sigma Aldrich Chemie, GMBH, Germany, EU). Before use, MTT was dissolved in phosphate buffered saline and ConA in culture medium. MTT and ConA solutions were sterile filtered (Flowpore, pore size 0.22 μm) before use. Radioactively labeled thymidine (^{3}H-TdR, Amersham, UK) was used for T cell activation experiments. Monoclonal mouse antibodies to human proliferating cell nuclear antigen, PCNA and polyclonal rabbit antihuman CD3, both cross reactive with respective rat antigens, and substrate, DAB

liquid, were purchased from Dako Cytomation (Denmark). RPMI-1640 liquid culture medium (PAA Laboratories, Austria, EU), supplemented with 5% (v/v) heat inactivated fetal calf serum (FCS) (PAA Laboratories, Austria, EU) and 20 g/mL gentamycine (complete medium) were used in cell culture experiments.

Animals and Treatment

Male Dark Agouti (DA) rats (from Institute for Biological Research "Sinisa Stankovic", Belgrade, Serbia) of 10-12 weeks old and weighing 200 g were used. Animals' treatment was carried out in adherence to the guidelines of the Ethical Committee of the Institute. They were maintained at 12 h photoperiod, under 21-24 °C temperature control, and water and food were available *ad libitum*. Four to six animals were randomly assigned to each treatment group. The backs of the animals were closely clipped of fur with electric animal clipper, and care was taken not to damage the skin. Warfarin was dissolved in endotoxin free saline at 100 μg/mL and applied to the skin (approximately 10-12 cm^2) in a volume of 100 μL once a day, for three consecutive days. In this way, rats received 0.8-1 μg of warfarin per cm^2 daily. Rats in the control group were treated with saline only. Oral ingestion was avoided by the application of warfarin to the upper portion of the animal's back, close to the neck, inaccessible to licking. After treatment, animals were caged individually. Skin samples were taken 24 h and 72 h after the last warfarin or saline application and processed for functional and histological studies.

Lipid Peroxidation

Warfarin-treated skin was taken 24 h and 72 h following the last application, trimmed free of subcutaneous tissue and cut to 1 cm^2 pieces, which were finely minced with scissors and homogenized in 0.25 mol/L saccharose-phosphate buffer (pH 7.2) by IKA T18 Basic Homogenizer (IKA Works Inc, Wilmington NC, USA). Skin homogenates were sonicated (3x15 seconds) on ice at 30% of maximum intensity amplitude by Bandelin Electronic UW 2070 sonicator (Bandelin Electronic, Berlin, Germany, EU). Lipid peroxidation was evaluated by the thiobarbituric acid reaction as described[27]. In brief, tissue homogenates were mixed 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 by using a spectrophotometer (Shimadtzu Corporation, Lakewood, USA). MDA content in skin homogenates was calculated by the reference to a standard curve constructed by known amounts of MDA. Data were expressed as nanomols of MDA/g of freshly explanted skin tissue and nanomols of MDA/g of protein.

Protein Assay

Bradford assay[28] was employed for the determination of protein content in skin homogenates.

Histology

Skin samples were taken 24 h and 72 h following the last warfarin application, fixed in 4% buffered formaldehyde (pH 6.9) and embedded in paraffin wax for sectioning at 5 μm. Masson trichrome-stained or Giemsa-stained histology slides were analyzed for changes in the epidermal cell layer or fibroblasts, respectively.

Immunohistochemistry

The presence of $PCNA^+$ and $CD3^+$ cells in skin was evaluated by immunohistochemical staining with antibodies to respective antigens on paraffin sections. An antigen retrieval procedure was undertaken by heating paraffin sections in microwave oven in 0.5 mol/L citrate buffer, pH 6.0. The anti-PCNA and anti-CD3 were used as primary antibodies at 1/100 dilution. Mouse IgG2a (Dako Cytomation) and rabbit immunoglobulin fraction (Dako Cytomation) were used as negative controls, as recommended by the manufacturer. The sections with specific antibodies and with irrelevant antibodies were visualized by EnVision™ kit (DakoCytomation, Denmark) with DAB as chromogen. To determine the number of $CD3^+$ cells, at least thirty high-power (x40) fields of digital photomicrographs of skin samples were examined in a blinded fashion to make the objective quantitative analysis possible.

Determination of Skin Viability

A colorimetric *in vitro* assay described by Klein et al.[29] in which MTT is metabolically reduced by tissue cell oxidoreductases to formazan end-product, was employed to estimate the viability of warfarin-treated skin. Warfarin-exposed or control dorsal skin was excised 24 h or 72 h following the

last application. Skin was cleared from subcutaneous tissue, cut into small pieces, around 1 mm^2, and each piece was organ cultured in 0.2 mL of culture medium. Immediately (fresh explants, 0 hour) or after 24 h, MTT was added (0.5 mg/ml of culture) and skin explants were incubated for 3h at 37 °C in a humidified atmosphere of 5% CO_2. Produced formazan was dissolved by overnight incubation of skin explants in 2-methoxyethanol. Extracted chromogen was quantified at 540 nm with spectrophotometry, with the ELISA 96-well plate reader (Shimadzu Corporation, Lakewood, USA). Results are expressed as absorbance (A540 nm) per g of tissue and as an index of MTT reduction (MTT index), i.e. the ratio of absorbance of solubilized formazan formed by 24-hour organ-cultured and freshly explanted skin.

Epidermal Cell Preparation and Culture

Skin was cut into small pieces and incubated in dispase (2.5 mg/mL) at 4 °C overnight, in order to separate epidermis from the 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. For assessment of the epidermal cell viability and survival, MTT reduction assay for granulocytes described by Oez et al.[30] was employed. Cells were added to wells ($1x10^5$ cells/well) with culture medium. MTT (with the final concentration of 0.5 mg/mL) was added immediately and incubated with cells for 3 h at 37 °C in a humidified atmosphere of 5% CO_2. Produced formazan was dissolved by overnight incubation in acidified SDS (10% SDS-0.01N HCl). Extracted chromogen was quantified with spectrophotometry at 540 nm by using ELISA 96-well plate reader (Shimadzu Corporation, Lakewood, USA).

Tumor Necrosis Factor-α (TNF-α) Production by Epidermal Cells from Warfarin-treated Skin

For TNF-α production, epidermal cells were cultured in 96-well plates with $1x10^5$ cells / well in a complete culture medium for 48 h. TNF-α content in epidermal cell-conditioned medium was measured by using eBioScience ELISA kit (eBioscience Inc, San Diego, CA, USA). Assay was performed according to the manufacturer's instructions. TNF-α titer was calculated by reference to a standard curve constructed by known amounts of recombinant TNF-α.

Epidermal Cell Costimulatory Activity Determination

T cell activation assay which examined the capacity of cells to additionally stimulate (costimulate) lectin Concanavalin A (ConA)-induced T lymphocyte activation/proliferation[31] was used to evaluate immunomodulatory function of epidermal cells from warfarin-treated skin. Thymic T lymphocytes depleted of endogenous accessory cells by nylon wool column separation (non-adherent T lymphocytes), proliferated weakly in response to ConA stimulation, and addition of epidermal cells as exogenous accessory cells enhanced their proliferation[31]. Non-adherent T lymphocytes ($5x10^5$/well) stimulated with ConA (2.5 μg/mL) were cultured with epidermal cells ($1x10^5$/well) for 3 days at 37 °C in a humidified atmosphere with 5% CO_2. During the last 8 hours of a 72-hour culture period, cultures were incubated with radioactively labeled thymidine (^{3}H-TdR, Amersham, UK) (1 μCi/well) to evaluate T cell proliferation. Activation/proliferation is expressed as counts per minute (c.p.m.).

Statistical Analysis

Results were expressed as mean values ± SD for each experimental animal group (with four to six animals). Significance was defined by Mann-Whitney U test, and *P*-values less than 0.05 were considered significant.

RESULTS

Effects of Warfarin on Metabolic Viability of Skin

To determine whether epicutaneous application of warfarin affects skin viability, MTT assay described for determination of metabolic activity of skin[29] was used (Figure 1). There were no significant changes in MTT reducing capacity between saline- and warfarin-treated fresh skin explants according to the A540nm values measured per gram of skin. However, when skin biopsies were organ-cultured in complete medium for 24 h, increased MTT reducing capacity was noted in skin tissue taken 72 h following warfarin exposure, compared to those from control group (Figure 1A). This resulted in increased MTT index at that time point (Figure 1B).

Lipid Peroxidation in Warfarin-treated Skin

To examine whether epicutaneous application of warfarin is accompanied by oxidative stress, malondialdehyde (MDA) levels were determined in

homogenates of warfarin-treated skin (Figure 2). As shown in Figure 2A, a tendency (P=0.052) to an increase in MDA skin content was noted 24 hours following warfarin application. Standardization of skin MDA to protein content revealed significant increase in MDA formation in warfarin-exposed skin at this time point (Figure 2B).

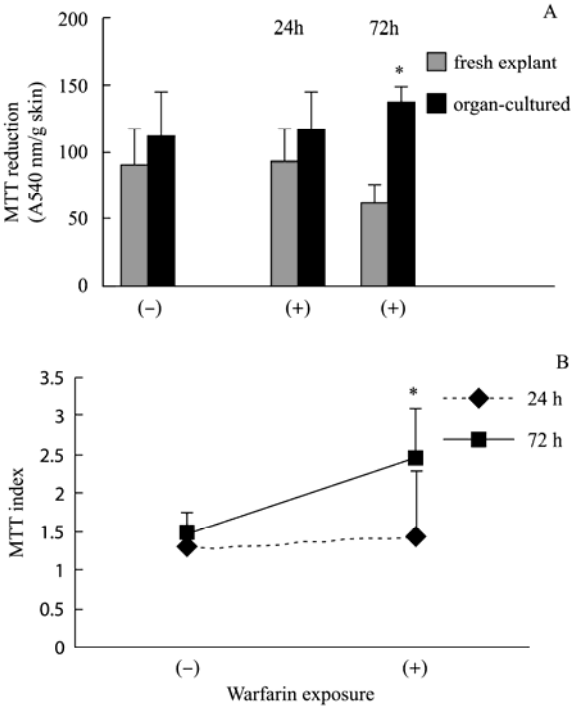

Figure 1. Metabolic viability of explants from control (-) and warfarin (+) exposed skin. (A) MTT reduction by freshly explanted skin (fresh explant) and following 24 h in culture (organ-cultured). (B) Index of MTT reduction. MTT reduction is expressed as absorbance at 540 nm per g of skin ± SD. MTT index was calculated as the ratio of absorbance of solubilized formazan formed by organ-cultured and freshly explanted skin ± SD. *denotes P<0.01 between warfarin-exposed and control skin.

Histology of Skin after Warfarin Application

The histological analysis of skin 24 h after warfarin application revealed morphological changes in epidermis, including compact layer fissuring, compared to basket wavy appearance of control epidermis (Figure 3A). On day three, large keratohyaline granules in the granular layer with cytoplasm vacuolization (intracellular edema), separation of the corneal layer from superficial keratinocytes and supragranular exudation were noted (Figure 3A). Pronounced cellularity of dermis was evident mainly owing to hypertrophic fibroblasts and their dark nuclei and cytoplasm, with irregular cell borders (Figure 3B). Capillary congestion with capillary wall edema were noted as early as 24 h following warfarin application, but were most pronounced at later time points (Figure 3B).

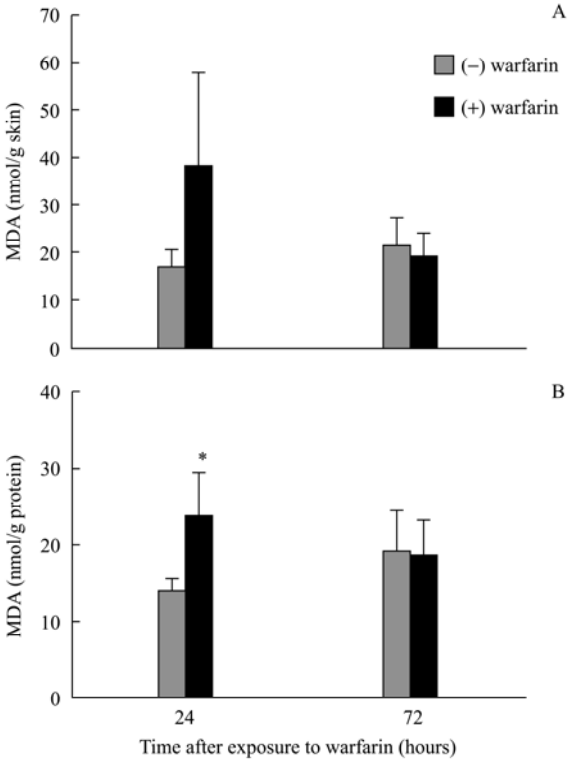

Figure 2. Malondialdehyde content in skin 24 h and 72 h after warfarin administration. (A) Skin MDA content per gram of skin. (B) Skin MDA to skin protein content. Results are expressed as the $\bar{x} \pm s$ of four to six animals per group. *denotes P<0.05 between warfarin-exposed and control skin.

Presence of PCNA$^+$ Cells in Warfarin-treated Skin

The immunohistochemical analysis revealed increased attendance of PCNA$^+$ cells in epidermis and hair follicles of warfarin-exposed skin (Figure 4). Increased numbers of PCNA$^+$ cells in the basal layer of epidermis and in upper epidermal layers, were noted 24 h following warfarin application. Presence of hypertrophic nuclei with a tendency to be unequal was noted at this time point. Individual basal keratinocytes with condensed, dark nucleus, resembling karyopicnosis, were occasionally noted

as well. More pronounced presence of $PCNA^+$ cells was noted 72 h following warfarin application, which was in a stratified position. No karyopicnosis in epidermal cells was found. Rare $PCNA^+$ cells were observed in the basal layer of epidermis of control skin. No $PCNA^+$ cells were found in dermis.

Presence of $CD3^+$ Cells in Warfarin-treated Skin

Increase in numbers of $CD3^+$ cells was detected immunohistochemically in warfarin-treated skin, but was statistically significant only 24 h following the last warfarin application (Figure 5). T cell tropism for epidermis and hair follicular epithelium was noted.

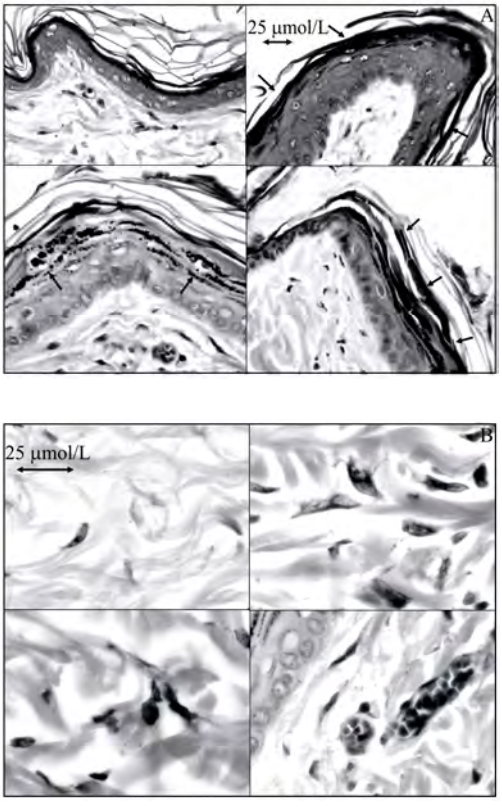

Figure 3. Histology of warfarin- exposed skin. (A) Changes in epidermis. Basket wavy appearance of keratin layer of control skin (up left). Absence of upper parts of keratin layer and compact layer fissuring (arrows, up right). Vacuolized cells with keratohyaline granules (arrows, down left). Supragranular exudation (arrows, down right). (B) Changes in fibroblast and capillary appearance. Control skin (up left). Hypertrophic fibroblasts (up right). Dark cells with irregular cell border (down left). Capillary congestion with capillary wall edema (down right).

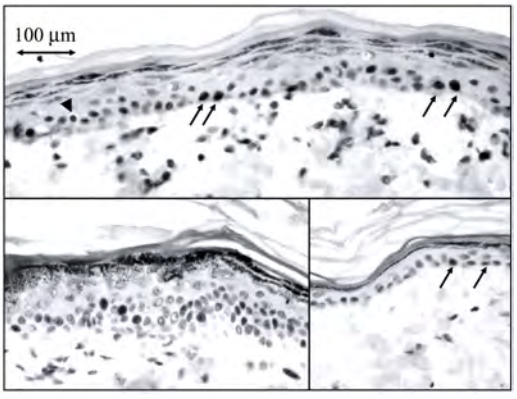

Figure 4. PCNA+ cells in warfarin- exposed skin. Increased numbers of $PCNA^+$ cells in basal layer of epidermis 24 h after warf arin application (arrows, up). Basal kerati nocyte with condensed, dark nucleus (arrow head, up). $PCNA^+$ cells in stratified position 72 h following warfarin application (dow n left). Rare $PCNA^+$ cells in basal layer of epid ermis of control skin (arrows, down right).

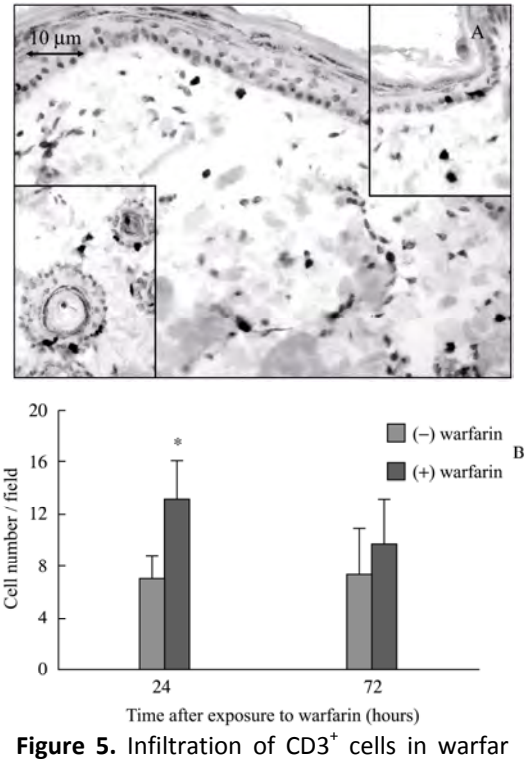

Figure 5. Infiltration of $CD3^+$ cells in warfar in-exposed skin. (A) $CD3^+$ cells in epidermis (upper insert) and in folicular epithelium (lower insert). (B) Numbers of $CD3^+$ cells. Results expressed as average numbers of positive cells counted at more than 30 high power fields ±SD. * denotes $P<0.05$ between warfarin-exposed and control skin.

Immune/Inflammatory Activity of Epidermal Cells from Warfarin-treated Skin

As the epidermis is the primary target of epicutaneous warfarin, some aspects of epidermal cell activity were determined. Warfarin did not affect epidermal cell viability in view of unchanged MTT reducing capacity of epidermal cells from warfarin-treated cells, compared to that in control cells (not shown). Epidermal cells isolated from skin three days following the last warfarin application produced more TNF-α, compared to the cells from control skin (Figure 6A). When costimulatory activity of epidermal cells from skin exposed to warfarin was tested in T cell activation/proliferation assay (Figure 6B), increased capacity of costimulation was noted in cocultures with epidermal cells from warfar in-expo sed skin compared to cells from control skin.

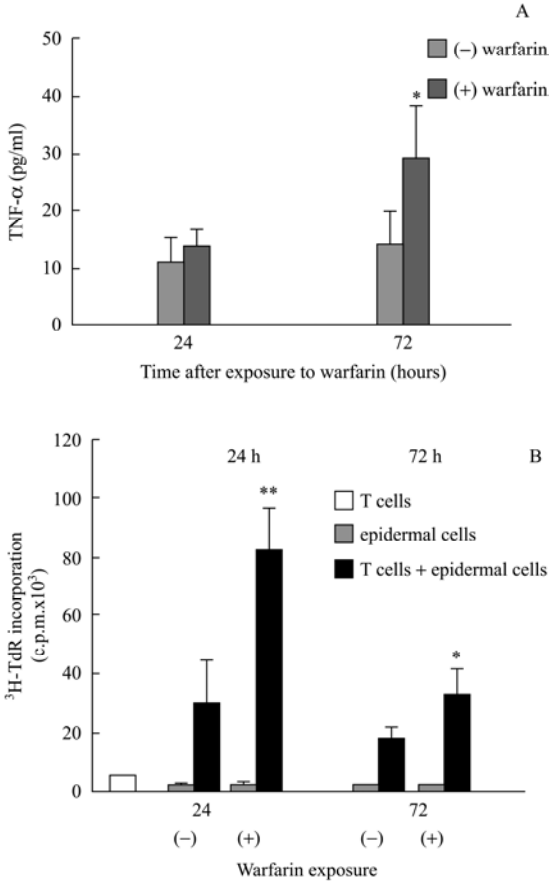

Figure 6. Immune/inflammatory activity of epidermal cells from warfarin-exposed skin. (A) Epidermal cells production of TNF-α. (B) Costimulatory activity of epidermal cells. Results are expressed as the $\bar{x} \pm s$ of four rats per group. * denotes $P<0.05$ and ** $P<0.01$ bet ween warfarin-exposed and control skin.

DISCUSSION

In this work, local effects of repeated epicutaneous application of anticoagulant rodenticide warfarin were examined with the aim to assess whether warfarin application was associated with tissue injury and inflammation.

Biochemical, histomorphological and immunohistochemical analyses of warfarin-treated skin revealed changes indicative of or related to tissue injury. Although MTT assay showed unchanged capacity of freshly explanted warfarin-treated skin to reduce metabolically this tetrazolium salt, warfarin cytotoxicity could be masked by inflammatory/immune activity noted in exposed skin. MTT assay is based on the activity of cellular oxidoreductases and thus prone to modulation by factors that affect their abundance and/or activity. In this sense, cytokine-mediated increase in MTT reduction was reported in some cells[32-33] as well as in explants of inflamed skin[34]. Increased MTT reducing capacity of skin explants from warfarin-exposed skin taken 72 h following warfarin application supports such assumptions.

An increased level of malondialdehyde, end-product of lipid peroxidation, indicates reactive oxygen species production, suggesting direct dermatotoxicity of epicutaneous warfarin. Presence of MDA in warfarin-treated skin reflects prooxidant activity of warfarin as suggested by our previous study of a single exposure of skin to this agent[21]. Morphohistological changes in warfarin-exposed skin depicted direct dermatotoxicity of warfarin. Increased exudation observed between keratin layers is most likely an indication of superficial keratinocyte damage that accumulates extra cytoplasmic liquid. Rough keratohyaline granules depict barrier reparative processes in exposed epidermis. Dark nuclei and cytoplasm with irregular cell border noted in dermal fibroblasts, suggest degenerative changes associated with necrosis. Absence of PCNA$^+$ cells in dermis implies the lack of reparative processes in these cells. Changes in fibroblasts are most likely due to a direct warfarin action, as percutaneous absorption of epicutaneous warfarin was noted in rats[35]. Vascular (capillary) changes noted in dermis of skin exposed to warfarin reflect reaction to connective/vascular tissue injury.

Increased attendance of PCNA$^+$ cells in warfarin-exposed skin suggests tissue-damaging properties of warfarin, as PCNA protein is a component of cell replication and repair machinery[36] and might be considered as an

indication of proliferative and reparative processes. Increased numbers of $PCNA^+$ cells probably reflect reparative processes secondary to skin damage induced by warfarin application, as shown for UV-irradiated human skin[37], where induction of PCNA protein is regarded as a part of skin DNA response to UV exposure[38].

Accumulation of $CD3^+$ cells (T lymphocytes) in warfarin-treated skin as well as immune/inflammatory activity of cells isolated from the epidermis of exposed skin demonstrated proinflammatory effects of epicutaneosly applied warfarin.

Increased production of TNF-α, cytokine known to influence various aspects of skin cell biology and activity[39], might account for, at least partly, presence of $CD3^+$ cells in warfarin-treated skin as shown in healthy or diseased skin[40-42]. Presence of $CD3^+$ cells in epithelium of hair follicle and epidermis of warfarin-exposed skin demonstrated tropism of T lymphocytes for these locations. Warfarin penetration to hair follicles might account for follicular T cell infiltration, while migration of $CD3^+$ cells to epidermis possibly relies on the warfarin-induced activity of epidermal cells from exposed skin.

Increased production of TNF-α by epidermal cells isolated from warfarin-exposed skin is in line with the data which demonstrated TNF-inducing activity of one of the coumarin derivatives, aurapten, by phagocyte cells[43]. In a broader sense, warfarin-induced epidermal cell production of TNF-α is in line with the data which showed that proinflammatory cytokines, such as TNF-α, are biological markers of keratinocyte response to chemicals[23]. Increased costimulatory activity of epidermal cells in ConA-stimulated T cell activation/proliferation assay demonstrated greater capacity of epidermal cells to function as accessory cells in T cell activation. Increased capacity of epidermal cells from warfarin-treated skin to stimulate T-cell activation and proliferation might be of relevance for skin infiltrating T cell activity and *vice versa*.

Observed immune/inflamatory activity in warfarin-treated skin might possibly represent an inflammation-based reparatory program triggered in skin by warfarin-induced tissue injury. Changes observed might have resulted from warfarin-induced skin inflammation, as some of inflammatory mediators have tissue damaging properties and might be introductory to inflammation-related pathological circuits in exposed skin.

Data presented in this study, are in line with the World Health Organization classification of warfarin and related hydroxycoumarins (coumachlor and coumatetralyl) as highly dangerous environmental agents[12], and thus of relevance for further information concerning toxicity of anticoagulant rodenticides. Rare studies in humans showed slight skin irritation in persons handling some coumarin-based rodenticides[12]. Some data showed that even after application of coumarin in ethanol or oil-in-water emulsion (that enhances skin penetration) on split-thickness human skin explants, 5% of applied coumarin remained in skin and was metabolically unchanged[44]. Such data pointed out the significance of cutaneous route of exposure. According to reports in which adverse effects of percutaneous warfarin exposure were described[13,15,45], the levels of skin exposure to warfarin varied with the content of warfarin in preparations as well as with frequency and duration of exposure. In one study, 30-minute exposure to 0.5% warfarin solution (every day for two weeks) during preparation of rat baites was described[46]. In another study, exposure to 0.025% warfarin once a week for several weeks (nonprofessional application of commercially available rodenticide preparation) was reported[13]. There was also a report on high-dose exposure (1.7% and 6.5% warfarin) in talcum powder[15].

Morphohistological and functional changes noted in skin following acute exposure to warfarin might be relevant to long-term warfarin exposure, rather than to short-term exposure. Although not directly comparable, data from clinical studies which showed cutaneous side effects of warfarin therapy including pruritic erythematous maculopapular eruptions[46-48], hemorrhagic necrosis of the skin[49-51], calcific panniculitis and small vessel calcification[52], livedo reticularis[53], and purple toes syndrome[50,54-55], proved skin to be a target tissue for warfarin. Given the range of warfarin effects on cell activity, either acute or chronic exposure, might prepare skin tissue for an intenser or suppressed response to subsequent physiological or pathological stimuli.

In conclusion, data presented in this study demonstrate that epicutaneous warfarin application results in tissue injury and that this is associated with immune/inflammatory activity in treated skin. These findings are conducive to recognition of skin as a target for toxic effects of this agent and contribute

to a better understanding of toxicity of this anticoagulant rodenticide.

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REFERENCES

1. Shearer MJ. Vitamin K and vitamin K-dependent proteins. Br J Haematol. 1990; 75, 156-62.
2. Furie B. Oral anticoagulant therapy. In: Hematology. Basic Principle and Practice (Hoffman R, Benz EJ, Shatill SJ, et al. Eds), A Harcourt Health Science Company, Churchill Livingstone, 2000; pp. 2040-6.
3. Price PA. Role of vitamin K-dependent proteins in bone metabolism. Annu Rev Nutr 1988; 8, 565-83.
4. Nakano T, Kawamoto K, Kishino J, et al. Requirement of gamma-carboxyglutamic acid residues for the biological activity of Gas6: contribution of endogenous Gas6 to the proliferation of vascular smooth muscle cells. Biochem J, 1997; 323, 387-92.
5. Yanagita M, Ishii K, Ozaki H, et al. Mechanism of inhibitory effect of warfarin on mesangial cell proliferation. J Am Soc Nephrol, 1999; 10, 2503-9.
6. Hafizi S, Dahlbäck B. Gas6 and protein S. Vitamin K-dependent ligands for the Axl receptor tyrosine kinase subfamily. FEBS J, 2006; 273, 5231-44.
7. Kulman JD, Harris JE, Xie L, et al. Identification of two novel transmembrane γ-carboxyglutamic acid proteins expressed broadly in fetal and adult tissues. Proc Natl Acad Sci USA, 2001; 98, 1370-5.
8. Manfioletti G, Brancolini C, Avanzi G, et al. The protein encoded by a growth arrest-specific gene (gas6) is a new member of the vitamin K-dependent proteins related to protein S, a negative coregulator in the blood coagulation cascade. Mol Cell Biol, 1993; 13, 4975-85.
9. Sherman S, Hall BD. Warfarin and fetal abnormality. Lancet, 1976; 1, 692.
10. Fiore CE, Tamburino C, Foti R, et al. Reduced axial bone mineral content in patients taking oral anticoagulant. South Med J, 1990; 83, 538-42.
11. Resch H, Pietschmann P, Krexner E, et al. Decreased peripheral bone mineral content in patients under anticoagulant therapy with phenprocoumon. Eur Heart J, 1991; 12, 439-41.
12. World Health Organization. Environmental Health Criteria, No. 175. Anticoagulant rodenticides. WHO, Geneva, 1995; 1-121.
13. Abell TL, Merigian KS, Lee JM, et al. Cutaneous exposure to warfarin-like anticoagulant causing an intracerebral hemorrhage: a case report. Clin Toxicol, 1994; 32, 69-73.
14. Farrar A, Cabral R. Recent epidemics of poisoning by pesticides. Toxicol Lett, 1995; 82/83, 55-63.
15. Martin-Boyer G, Khanh NB, Linh PH. Epidemic of hemorrhagic disease in Vietnamese infants caused by warfarin-containing talc. Lancet, 1983; 1, 230-2.
16. DiPalma JR. Human toxicity from rat poisons. Am Fam Physician, 1981; 24, 186-9.
17. Spiller HA, Gallenstein GL, Murphy MJ. Dermal absorption of a liquid diphacinone rodenticide causing coagulaopathy. Vet Hum Toxicol, 2003; 45, 313-4.
18. Huckle KR, Morrison BJ, Warburton PA. The percutaneous fate of the rodenticide flocoumafen in the rat: role of non-biliary intestinal excretion. Xenobiotica, 1989; 19, 63-74.
19. Beckley-Kartey SA, Hotchkiss SA, Capel M. Comparative in vitro skin absorption and metabolism of coumarin (1,2-benzopyrone) in human, rat, and mouse. Toxicol Appl Pharmacol, 1997; 145, 34-42.
20. Rheins A. What's new in cutaneous toxicity? J Toxicol Cut Ocular Toxicol, 1992; 11, 225-38.
21. Kataranovski M, Prokić V, Kataranovski D, et al. Dermatotoxicity of epicutaneously applied anticoagulant warfarin. Toxicology, 2005; 212, 206-18.
22. Kataranovski M, Kataranovski D, Zolotarevski L, et al. Epicutaneous exposure to anticoagulant rodenticide warfarin modulates local skin activity in rats. Cutan Ocul Toxicol, 2007; 26, 1-13.
23. Luster MI, Wilmer JI, Germolec DR, et al. Role of keratinocyte-derived cytokines in chemical toxicity. Toxicol Lett, 1995; 82/83, 471-6.
24. Shvedova AA, Kommineni C, Jeffries BA, et al. Redox cycling of phenol induces oxidative stress in human epidermal keratinocytes. J Invest Dermatol, 2000; 114, 354-64.
25. Shvedova AA, Kisin E, Kisin J, et al. Elevated oxidative stress in skin of B6C3F1 mice affects dermal exposure to metal working fluid. Toxicol Ind Health, 2001; 16, 267-76.
26. Sullivan JB, Levin RJ, Bangert JL, et al. Clinical dermatotoxicology. In: Clinical Environmental Health and Toxic Exposures (Sullivan J.B., Krieger G.R., Eds.), 2001; pp.182-206. Williams & Wilkins, PA Lippincot.
27. Villacara A, Kumami K, Yamamoto T, et al. Ishemic modification of cerebrocortical membranes: 5-hydroxytryptamine receptors, fluidity, and inducible in vitro lipid peroxidation. J Neurochem, 1989; 53, 595-601.
28. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem, 1976; 72, 248-54.
29. Klein MB, Shaw D, Barese S, et al. A reliable and cost-effective in vitro assay of skin viability for skin banks and burn centers. J Burn Care Rehabil, 1996; 17, 565-70.
30. Oez S, Platzer E, Welte K. A quantitative colorimetric method to evaluate the functional state of human polymorphonuclar leukocytes. Blut, 1990; 60, 97-102.
31. Stošić-Grujičić S, Lalošević D, Lukić ML. Modulatory effects of glucocorticoids on immunoregulatory functions of epidermal cells. Int J Immunopharmacol, 1987; 9, 577-85.
32. Berridge MV, Tan AS. Characterization of the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): subcellular localization, substrate dependance, and involvement of mitochondrial electron transport in MTT reduction. Arch Biochem Biophys, 1993; 303, 474-82.
33. Jabbar SA, Twentyman PR, Watson JV. The MTT assay underestimates the growth inhibitory effects of interferons. Br J Cancer, 1989; 60, 523-8.
34. Varljen T, Prokić V, Nikolić T, Kataranovski M (2000). Evaluation of skin functional state by MTT test in organ culture. In: Trauma, Shock, Inflammation and Sepsis. Pathophysiology Immune Consequences and Therapy (Faist E. Eds.), pp. 667-71. Monduzzi Editore. Internationale Proceedings Division,
35. Kataranovski M, Mirkov I, Vranković J, et al. Percutaneous toxicity of anticoagulant warfarin in rats. Cutan. Ocul Toxicol, 2008; 27, 29-40.
36. Kelman Z. PCNA: structure, functions and interactions. Oncogene, 1997; 14, 629-40.
37. Zeng XR, Jiang Y, Zhang SJ, et al. DNA polymerase delta is involved in the cellular response to UV damage in human cells. J Biol Chem, 1994; 269, 13748-51.
38. Hall PA, McKee PH, Menage HD, et al. High levels of p53

protein in UV-irradiated normal human skin. Oncogene, 1993; 8, 203-7.
39. Williams IR, Kupper TS. Immunity at the surface: homeostatic mechanisms of the skin immune system. Life Sci, 1996; 58, 1485-507.
40. Banno T, Gazel A, Blumenberg M. Effects of tumor necrosis factor-alpha (TNF alpha) in epidermal keratinocytes revealed using global transcriptional profiling. J Biol Chem, 2004; 279, 32633-42.
41. van Hogerlinden M, Rozell BL, Toftgard R, et al. Characterization of the progressive skin disease and inflammatory cell infiltrate in mice with inhibited NF-kappaB signaling. J Invest Dermatol, 2004; 123, 101-8.
42. DiNuzzo S, Sylva-Steenland MR, de Rie MA, et al. UVB radiation preferentially induces recruitment of memory CD4+ T cells in normal human skin: long-term effect after a single exposure. J Invest Dermatol, 1998; 110, 978-81.
43. Tanaka T, Sugiura H, Inaba R, et al. Immunomodulatory action of citrus auraptene on macrophage functions and cytokine production of lymphocytes in female BALB/c mice. Carcinogenesis, 1999; 20, 1471-6.
44. Yourick JJ, Bronaugh RL. Percutaneous absorption and metabolism of coumarin in human and rat skin. J Appl Toxicol, 1997; 17, 153-8.
45. Fristedt B, Sterner N. Warfarin intoxication from percutaneous absorption. Arch Environ Health, 1965; 11, 205-8.
46. Sheps SG, Gifford RW. Urticaria after administration of warfarin-sodium. Am J Cardiol, 1959; 3, 118-20.
47. Schiff BL, Pawtucket RI, Kern AB. Cutaneous reaction to anticoagulants. Arch Dermatol, 1968; 98, 136-7.
48. Kwong P, Roberts P, Prescott SM, et al. Dermatitis induced by warfarin. JAMA, 1978; 239, 1884-5.
49. Eby S. Warfarin-induced skin necrosis. Hematol./Oncol. Clin. North Am, 1993; 1, 1291-9.
50. Sallah S, Thomas DR, Roberts HR. Warfarin and heparininduced skin necrosis and the purple toes syndrome: infrequent complications of anticoagulant therapy. Thromb Haemost, 1997; 78, 785-90.
51. Hermes B, Haas N, Henz BM. Immunopathological events of adverse cutaneous reactions to coumarin and heparin. Acta Derm Venereol, 1997; 77, 35-8.
52. Coates T, Kirkland GS, Dymock RB, et al. Cutaneous necrosis from calcific uremic arteriolopathy. Am J Kidney Dis, 1998; 32, 384-91.
53. Park S, Schroeter AL, Park YS, et al. Purple toes and livedo reticularis in a patient with cardiovascular disease taking coumadin. Arch Dermatol, 1993; 129, 775-80.
54. Akle CA, Joiner CL. Purple toe syndrome. J. R Soc Med, 1981; 74, 219.
55. Hyman BT, Landas SK, Ashman RF, et al. Warfarin-related purple toes syndrome and cholesterol microembolization. Am J Med, 1987; 82, 1233-7.