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ORIGINAL ARTICLES

Production of TNF-Alpha by Skin Explants of Dinitrochlorobenzene-challenged Ears in Rats: a Model for the Evaluation of Contact Hypersensitivity

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Background. *Contact hypersensitivity (CHS) is a local inflammatory response of the skin following challenge of hapten-sensitized animals. It is the consequence of cell infiltration of derm and the release of inflammation mediators, among which Tumor necrosis factor-alpha (TNF-α) is one of the most important factors. The intensity of the inflammation could be quantified by ear swelling which is the classical manifestation of the reaction. This study was testing the working hypothesis that levels of TNF-α in skin organ culture medium should correlate with the intensity of CHS reaction measured in vivo by ear swelling assay, and with the density of dermal infiltrate in ear skin samples. In order to test the working hypothesis, the intensity of inflammatory reaction following challenge was evaluated by classical measurements of ear swelling, by the determination of TNF-α levels in culture fluids of ear skin following epicutaneous application of dinitrochlorobenzene (DNCB) into the ears of sensitized animals.* ***Methods.*** *Animal model of CHS reaction to DNCB in Albino Oxford rats was used as described. Ear swelling was quantified in percentage terms as the difference in thickness between the challenged and nontreated ears of the same animal. Dermal infiltrate density in histopathologically analyzed samples of ear skin was evaluated by computer-assisted image analysis. Ear skin samples were cultured in standard medium for 24 h, and TNF-α concentration in the conditioned medium was subsequently determined with ELISA test.* ***Results.*** *Dose-dependent increase in the density of the dermal infiltrate and in TNF-α in CM were noted following the application of 0.65%, 1.3% and 2.6% of DNCB to the ears of previously sensitized rats. The correlation between ear swelling and the levels of TNF-α ($r=0.933$, $p<0.001$) in CM, and between ear swelling and dermal infiltrate density ($r=0.916$, $p<0.001$) was found. Correlation was also found between the density of the dermal infiltrate and the levels of TNF-α ($r=0.865$, $p<0.001$).* ***Conclusion.*** *Presented data suggested that skin-organ culture system and the quantification of inflammatory mediators might be used for the evaluation of contact hypersensitivity reaction and its intensity.*

K e y w o r d s : **dermatitis, contact; tumor necrosis factor; dinitrochlorobenzene; ear; edema.**

Introduction

Contact hypersensitivity (CHS) is a T cell-mediated inflammatory response following skin challenge of hapten-sensitized animals. Afferent phase of contact hypersensitivity response (sensitization phase), is initiated by epicutaneous application of hapten to dorsal or abdominal trunk skin, and is characterized by the activation and the proliferation of hapten-specific T lymphocytes in regional lymph nodes and the occurence of effector cells in lymph nodes and spleen (1). In the efferent phase, after repeated epicutaneous application of the sensitizer to the skin of the ear, primed T

lymphocytes are recruited to the site of challenge where they produce a variety of inflammatory mediators, amplifying a background inflammatory response into a more vigorous process. It is the classical manifestation of contact hypersensitivity, measured as „ear swelling". Ear swelling is early-recognized skin response to hapten application, characterized histologically by dermal cell infiltration (2). The magnitude of ear swelling reflects the intensity of local inflammatory response, determined largely by the presence and the activity of cytokines and adhesion molecules which govern leukocyte extravasation and effector functions *in situ* (3–6)**.**

Among cytokines involved in CHS reaction, tumor necrosis factor-alpha (TNF-α) seemed to play a major role in the elicitation of CHS. From the data obtained in mouse model regarding contact hypersensitivity to trinitrochlorobenzene (TNCB) it was evident that local administration of anti-TNF-α antibodies disabled ear swelling (3) and that high correlation existed between the intensity of reaction and TNF-α mRNA expressed in the skin (3). By the same procedure, only a complementary role of IFN-γ, IL-2, GM-CSF and IL-3 in the expression of CHS was demonstrated.

Short-term culture of skin explants is a new experimental *in vitro* system designed to simulate *in vivo* conditions (7). The development of skin organ culture techniques provided the investigation of various aspects of normal and pathological skin biology, including maintenance of homeostasis, inflammation, effects of chemical and physical agents, and cell migration, as well (7). Cutaneous inflammation was investigated mainly by monitoring the presence of various soluble biochemical and inflammatory/immunoregulatory mediators released from skin explants in culture fluids. By this system various mediators were collected and defined as relevant for cutaneous inflammation, including biologic active amines, proteolytic enzymes, various serum proteins and cytokines (8–11).

In this study organ culture of rat skin was used to evaluate contact hypersensitivity to hapten dinitrochlorobenzene (DNCB). Skin response following local application of DNCB to the ears of the sensitised animals was investigated in previously described experimentally induced contact hypersensitivity rection in rats (12, 13). The working hypothesis was that levels of TNF-alpha in skin organ culture medium correlated with the intensity of CHS reaction measured *in vivo* by ear swelling assay, and with density of dermal infiltrate. In order to test the working hypothesis, the intensity of inflammatory reaction following challenge was evaluated by classical measurements of ear swelling, by quantitative histology (i.e. determinations of dermal infiltrate density by computer-assisted image analysis) and by the determination of TNF-α levels in culture fluids of ear skin following epicutaneous application of DNCB to the ears of sensitized animals. The relationship between *in vitro* determined parameter (TNF-α) and *in vivo* parameters (ear swelling and histology) of ear skin inflammation was examined, too.

Methods

Chemicals and reagents

In order to cause the reaction of contact hypersensitivity *in vivo* 1-chloro-2,4-dinitrochlorobenzene (DNCB - BDH Chemicals LTD, England) was dissolved in vehicle (acetone in olive oil, AOO, 4:1) which was used as a base and was applied in the control group animals.

Animals

All experiments were done in adherence to the NIN guidelines for the use of experimental animals, with the permission of the Ethical Comittee of our Institute. Inbred male Albino Oxford (AO) rats 3 months of age (Farm for Experimental Animals, Military Medical Academy, Belgrade, Yugoslavia), were housed in air-conditioned rooms at 25^{o}C on a 12-h light/dark cycle. Animals were provided with pelleted food and water *ad libitum*. During the experiment animals were caged individually.

Contact sensitization and ear swelling response

Groups containing six animals each received 100 μl of DNCB (2%, 4% or 8% w/v in vehicle) or an equal volume of vehicle (4:1 acetone-olive oil) on the shaved area of the back for two consecutive days. Three days later, rats were challenged by an application of 50 μl of three times lesser concentration of DNCB than the one used in the sensitization phase (i.e. 0.66%, 1.3% and 2.6%) on the dorsal skin of the left ear. Contact hypersensitivity reaction was assessed by measuring ear thickness with engineer micrometer 24 hours after challenge. The response was quantified as the difference in thickness between challenged and nontreated ears of the same animal according to the formula (C–N)/N×100, where C represented thickness of the challenged ear and N the thickness of nontreated ear. The response was expressed as the percent of increase of ear thickness.

Histology

Cartilage-free halves of exposed ears were taken 24 h after the elicitation of CHS reaction and were fixed in 10% (pH 6.9) formaldehyde for routine histological staining with haematoxyllin and eosin (H&E) in order to examine epidermal changes and the density of the dermal infiltrate. H&E-stained sections from the central areas of ear skin were compared among experimental groups. The density of the dermal infiltrate was measured by the computer-assisted image analysis system. After randomizing and coding the slides, video images were taken using the Sony 1/3 inch camera connected to the Olympus light microscope and the images were transmitted to PC. The image capturing and image analysis software was „MIKRO" (Laboratory for the computer systems, Institute „Mihajlo PUPIN", Belgrade). Five fields, which covered the entire area of the section, corresponding to 0.4 mm^2, were used for quantification and the mean values were statistically analyzed.

Skin Organ Culture

Exposed ears were taken 24 h after the elicitation of CHS, split into ventral and dorsal halves and submerged in 1.5 ml of culture medium in the wells of 24-well-culture plates. After 24h at 37°C, the conditioned medium (CM) was collected (7). TNF-α concentrations in CM were determined by commercially available ELISA test for rat TNF-α (Biosource Int., Camarillo, CA, USA).

Statistical analysis

Results were expressed as the mean value ±SD for each experimental animal group (6 animals). Statistical significance was determined by the Mann-Whitney test.

Correlation test was used for the evaluation of correlation between values and was expressed as r-index. Differences were considered significant at $p<0.05$, and highly significant at $p<0.01$.

Results

Ear swelling response and skin histological analysis following challenge of ears of sensitized animals

Contact hypersensitivity in AO rats was induced by the application of DNCB on the dorsal trunk of rats during two consecutive days (sensitization phase) and was challenged three days later by local epicutaneous application of DNCB on the left ear (elicitation phase). Control animals were treated with vehicle solely during sensitization and elicitation.

Dose dependent increase in ear swelling was demonstrated following the application of various concentrations of DNCB in the induction and the elicitation phase (Figure 1). This increase was accompanied by the dose-dependent increase of the inflammatory response in the exposed skin. Epidermal changes, similar to the familiar picture of allergic contact dermatitis, including vacuolisation of the basal layer and spongiosis with the formation of microvesicles at dermo-epidermal junction were seen (Figure 2). Superficial crusts

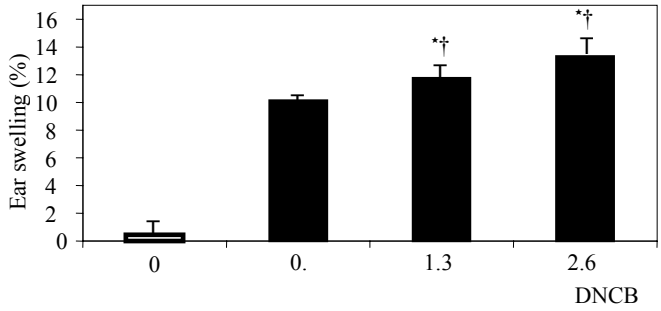

Fig. 1 – Ear swelling assay in the elicitation of CHS reaction. Values are expressed as the mean value of the percentage of increase in ear thickness in each treatment group ± standard deviation. * $p<0.05$ *vs.* 0%; † $p<0.05$ *vs.* 0.66% DNCB.

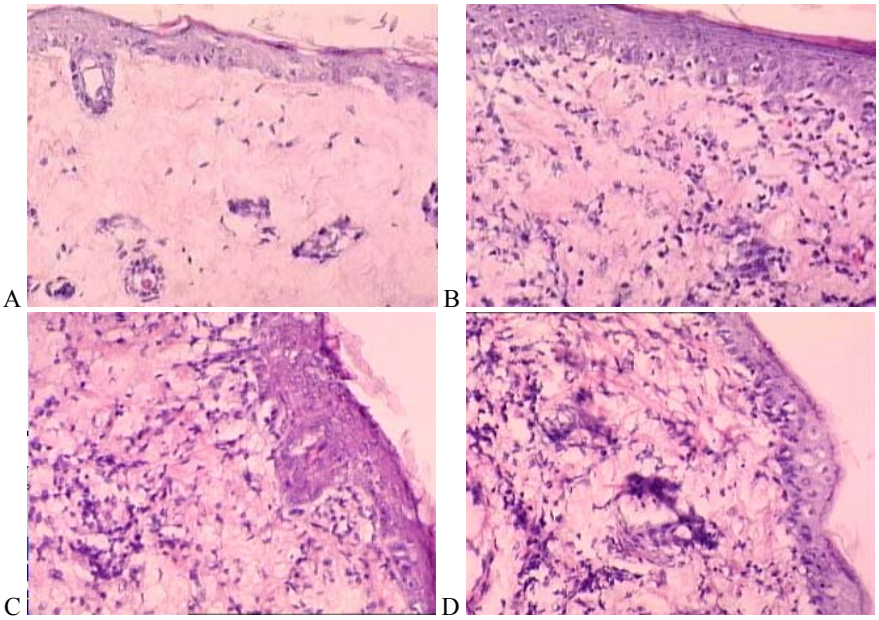

Fig. 2 – Histopathologic changes in ear skin samples in the elicitation phase of CHS reaction. A. vehicle: no changes; B. 0.66 % DNCB: hyperkeratosis, vacuolar degeneration of the basal epidermal layer, lymphocitic infiltrate in dermis. C. 1.33% DNCB: pronounced hyperkeratosis, microvesicles and bullae at dermo-epidermal junction, crusts at epidermal surface, diffuse mixed dermal infiltrate, dermal edema; D. 2.66% DNCB: hyperkeratosis, vacuolar degeneration of the basal epidermal layer, lymphocyte exocytosis, diffuse mixed dermal infiltrate and pronounced dermal edema. (H&E, ×400).

were the most prominent in the group of animals sensitized with 8% DNCB and challenged with 2.44% DNCB (8%/2.44% regimen group), less prominent in 4%/1.6% DNCB sensitization/chalenge regimen, and subtle in 2%/0.3% hapten group of treated animals. All of the described epidermal and dermal changes were absent in control groups treated only with AOO. The density of the dermal infiltrate, measured by the computer-assisted image analysis system, was gradually increased in the dose-dependent way (Figure 3A). Histological changes coincided with the increase in ear swelling and the correlation between ear swelling and dermal cell infiltration was noted (Figure 3B). These findings were coherent with the reported histological analysis.

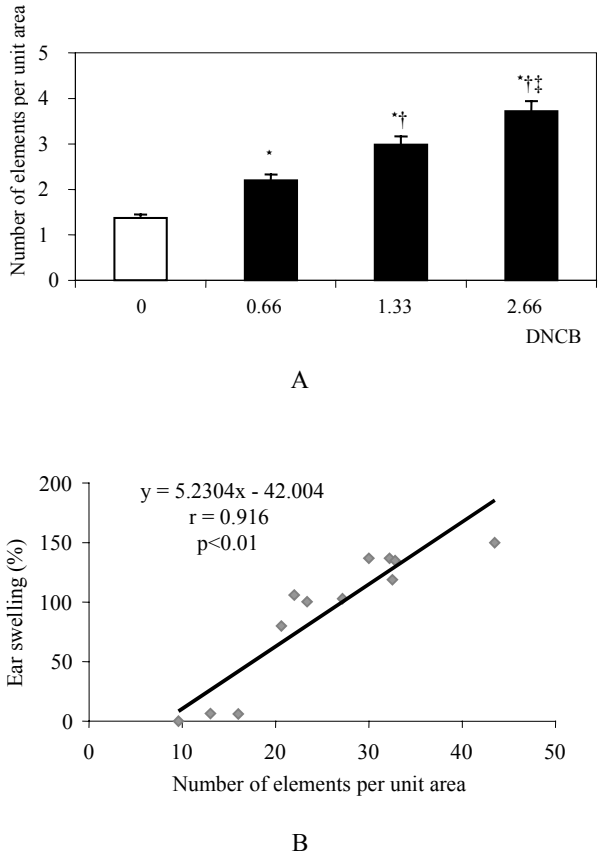

Fig. 3 – A. Quantification of cell density in dermal infiltrate in the elicitation phase of CHS reaction by computer-assisted image analysis system. ⋆ p<0.05 *vs.* 0%, † p<0.05 *vs.* 0.66% DNCB i ‡ p<0.05 *vs.* 1.33%; B. Regression curve between values of the dermal infiltrate density and ear swelling assay. Significance at 0.01 was considered highly significant.

Measurement of TNF-α concentration in culture fluids of ear skin explants

TNF-α was determined in the conditioned medium by commercial ELISA during 24 hours period. Significantly increased levels of TNF-α were noted in CM following application of 0.66%, 1.3% or 2.6% DNCB (43.9 ± 2.6 pg/ml, 48.5 ± 3.1 pg/ml and 50.4 ± 4.9 pg/ml, respectively) compared to 25.2 ± 1.1 pg/ml in CM of control, vehicle treated ears (Figure 4A). High correlation was noted between the levels of TNF-α and the intensity of ear swelling (r=0.933, p<0.001) (Figure 4B). Correlation also existed between TNF-α and the density of the dermal infiltrate (r=0.865, p<0.001) (Figure 4C).

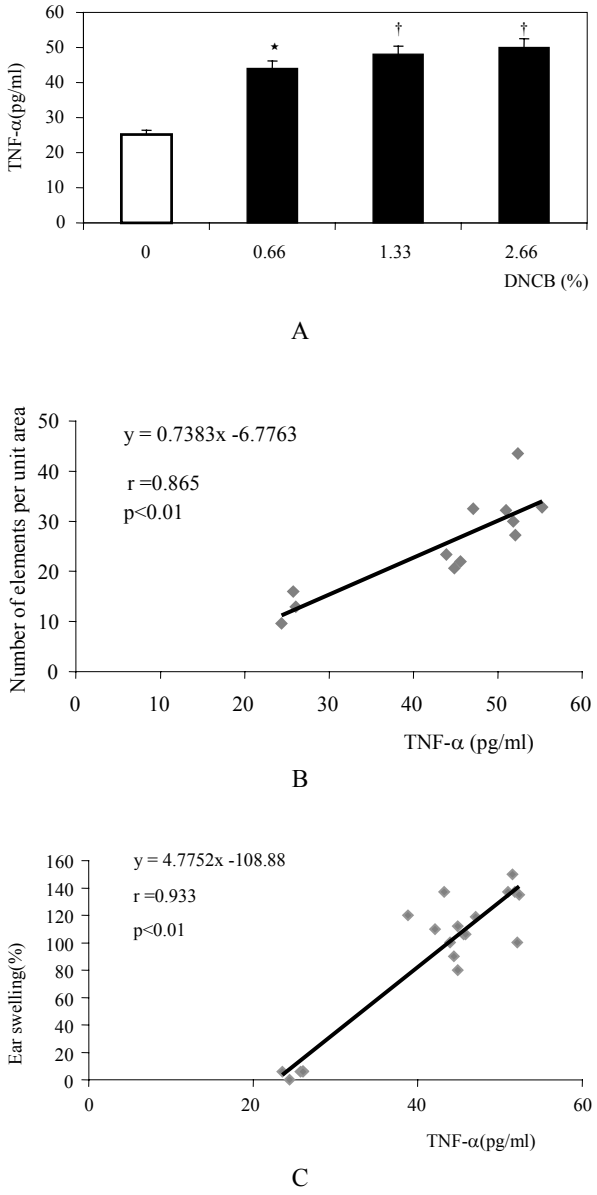

Fig. 4 – A.TNF-α levels in culture fluid of organ-cultured rat ear skin following topical application of various doses of DNCB. Values are expressed as pg of TNF-α/ml of culture fluid determined by commercially available ELISA test. ⋆ p<0.05 *vs.* 0%, † p<0.05 *vs.* 0.66% DNCB B. Regression curve between values of the TNF-α concentration and ear swelling assay. C. Regression curve between values of the TNF-α concentration and dermal infiltrate density. Significance at 0.01 was considered highly significant.

Discussion

Contact hypersensitivity (CHS) is a local inflammatory response of the skin following challenge of sensitized animals. Ear swelling is the earliest recognized manifestation of contact hypersensitivity characterized by dermal cell infiltration (2) of T cells, granulocytes, monocytes/macrophages, and Langerhans cells (14). It is based largely on the activity of skin cells which modulate skin microenvironment facilitating extravasation of inflammatory leukocytes through the release of cytokines. In the derm, leukocytes produce a variety of inflammatory mediators, amplifying the local response into vigorous inflammatory process expressed as ear swelling. In accordance with the abundance of data obtained in mouse models of CHS, following the administration of DNCB, a dose-dependent increase in ear swelling was noted in the rat model of CHS and coincided with the dose-dependent increase in cell infiltrate density. The application of DNCB increasing doses during the elicitation of CHS resulted also in the dose-dependent increase in levels of TNF-α in the conditioned medium of cultured ear skin explant. These data corresponded with other studies which demonstrated the employment of skin-organ-culture system for the detection of various inflammatory mediators produced and released from skin explants in culture fluids in various models of skin inflammation (7–11).

The rise in TNF-α protein in the conditioned medium of challenged ears might had resulted from skin cells (keratinocytes, dendritic cells) production of the cytokine, as described in mouse models of CHS (Piguet et al. 1991), but also from the infiltrating cells. Activated monocytes/macrophages are known as potent producers of TNF-α and along with the granulocyte TNF production (15) could as well contribute to skin-production of this cytokine. High correlation demonstrated between the degree of ear swelling and TNF-α released by skin of DNCB-treated ears into culture medium correlated with *in vivo* findings which suggested the involvement of skin-derived TNF-α in expression of CHS. Among cytokines TNF-α seemed to play the major role in the elicitation of CHS, as local administration of anti-TNF-α antibody before ear challenge restrained the elicitation of CHS measured by ear thickness. The significant role of TNF-α in CHS expression was further supported by the correlation between the intensity of CHS and TNF-α mRNA expression in the skin of hapten-treated ear in the elicitation of CHS to TNCB in mice (3). Data presented in this paper demonstrate that the measurement of TNF-α protein in the conditioned medium of skin explants could be employed as an *in vitro* approach for the evaluation of CHS expression. As such, these data also correlate with the use of skin explant technique as an *in vitro* method for the evaluation of contact sensitization (16).

Conclusion

Data presented in this paper demonstrate the evaluation of CHS response by *in vitro* measurement of TNF-α, cytokine which relevance for CHS was documented *in vivo*. One aspect of skin inflammation assessment in CHS expression by TNF-α should be mentioned. Namely, skin organ culture was recently recommended as a new experimental system suitable for studying and testing the effects of various physical, chemical and biological agents on skin (17). As CHS is frequently employed in the evaluation of various agents immunotoxicity (18, 19), in these investigations skin-organ culture system might be used as an accompanying test (with classical ear swelling/histology data). By using this culture system, regarding dermal toxicity of chemicals, more quantitative informations could be obtained.

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A p s t r a k t

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PRODUKCIJA FAKTORA NEKROZE TUMORA-α U ORGANO-KULTURI EKSPLANTATA KOŽE UVA PACOVA POD DEJSTVOM DINITROHLOROBENZOLA: MODEL ZA PROCENU KONTAKTNE PREOSETLJIVOSTI

Cilj. Reakcija kontaktne preosetljivosti (RKP) je lokalna inflamatorna reakcija kože na ponovljeni kontakt sa haptenom. Posledica je ćelijske infiltracije derma i oslobađanja medijatora inflamacije, a jedan od najvažnijih je faktor nekroze tumora-α (TNF-α). Klasična manifestacija kontaktne preosetljivosti je otok uva. U radu je testirana radna hipoteza da se koncentracija TNF-α u medijumu u kome su kultivisani eksplantati kože uva nakon RKP povećava sa povećanjem koncentracije nanetog haptena, i da su te promene u korelaciji sa promenama u otoku uva, koji predstavlja *in vivo* meru intenziteta RKP, kao i sa promenama u gustini infiltrata u dermu. Cilj rada bio je određivanje koncentracije TNF-α u medijumu u kome su kultivisani eksplantati kože uva tokom RKP na dinitrohlorobenzol i poređenje promena u koncentraciji TNF-α sa promenama u debljini otoka uva i gustini ćelijske infiltracije u dermu. **Metode.** Korišćen je ranije opisan model RKP na dinitrohlorobenzol (DNCB) kod pacova Albino Oxford soja. Veličina otoka uva izražavana je kao procenat zadebljanja levog u odnosu na desno uho. Uzorci kože uva patohistološki su analizovani, a gustina ćelijskog infiltrata u dermu merena je kompjuterskom obradom slike. Eksplantati kože kultivisani su tokom 24 časa, a u medijumu je zatim određivana koncentracija TNF-α ELISA-om. **Rezultati.** Utvrđeno je dozno-zavisno povećanje koncentracije TNF-α u medijumu i dozno-zavisno povećanje gustine infiltrata u dermu posle aplikacije 0,6%, 1,3% i 2,6% dinitrohlorobenzola na uho ranije senzibilisanih životinja. Osim korelacije između veličine otoka uva i gustine infiltrata u dermu (r=0,916, p<0,001), utvrđena je i korelacija između veličine otoka uva i koncentracije TNF-α u medijumu (r=0,933, p<0,001), kao i između gustine infiltrata u dermu i koncentracije TNF-α (r=0,865, p<0,001). **Zaključak.** Kultivacija eksplantata kože i merenje inflamatornih medijatora u medijumu za kultivaciju mogli bi da se koriste za dokazivanje inflamacije kože tokom reakcije kontaktne preosetljivosti, kao i za merenje njenog intenziteta.

K lj u č n e r e č i : dermatitis, kontaktni; faktor nekroze tumora; dinitrohlorbenzol; uvo; edem.