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CROATICA CHEMICA ACTA
CCACAA **78** (1) 91–98 (2005)

ISSN-0011-1643
*CCA*-2983
*Original Scientific Paper*

# A New Method for Detection of HNE-histidine Conjugates in Rat Inflammatory Cells

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RECEIVED JANUARY 8, 2004; REVISED OCTOBER 6, 2004; ACCEPTED OCTOBER 20, 2004

Oxidative stress, excessive production of reactive oxygen species, is considered an important part of different disorders, as well as of physiological processes (inflammation). The difference between physiological and pathological oxidative stress is often the occurrence of lipid peroxidation and its final toxic products, among which is 4-hydroxy-2-nonenal (HNE), a reactive aldehyde that forms protein conjugates. The aim of this study was to determine the distribution of HNE-histidine conjugates in leukocytes during systemic inflammation. We used genuine monoclonal antibodies against HNE-histidine conjugates for immuno-cytochemical, immuno-histochemical and immuno-electronmicroscopical analyses of HNE in inflammatory cells. Spleen tissue, leukocytes from blood and macrophages from the peritoneum of rats intraperitoneally (i.p.) injected with micronized zeolite (MZ) were analyzed. HNE-histidine conjugates were predominantly detected near cell membranes, phagosomes and macrophage granules. Immunodetection of HNE-histidine conjugates may be used as an analytical immunochemical method to study HNE formation in pathological and physiological processes and for pathomorphological diagnostic procedures.

*Keywords*
4-hydroxy-2-nonenal
oxidative stress
leukocytes
immunogold method

## INTRODUCTION

Oxidative stress is a balance shift of oxido-reductive reactions to oxidation, resulting in excessive production of reactive oxygen species (ROS).[1] ROS are normally formed in small quantities during metabolic processes.[2,3] They are also intensively produced by phagocytes during inflammation as a mechanism of defense against potentially harmful macromolecular agents and cells.[4,5] Hydroxyl radical is one of the most harmful ROS because of its high reactivity and instability and it is the only ROS that has enough energy to cause lipid peroxidation.[6,7] Overproduction of ROS is cytotoxic and damages macromolecules (DNA, proteins, sugars and lipids).[8]

During intense oxidative stress ROS damage polyunsaturated fatty acids (PUFA) causing a chain reaction of lipid peroxidation, resulting in the destruction of biomem-

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branes. Final products of lipid peroxidation are reactive aldehydes such as 4-hydroxyalkenals and other similar α,β-unsaturated aldehydes that also act as inflammatory mediators.[9,10,11]

A great number of these aldehydes have been isolated from biological samples, where they may promote and reinforce cell damage induced by oxidative stress.[12,13,14,15,16,17] Lipid-derived aldehydes are more stable than ROS and can therefore diffuse to targets far from the initial oxidative injury. Among these aldehydes, of particular biochemical and biomedical relevance is 4-hydroxy-2-nonenal (HNE), denoted the »second toxic messenger of free radicals«.[8,18,17]

HNE has 3 functional groups: an aldehyde group (C=O), a carbon-carbon double bond (C=C) and a hydroxyl group (OH). Which of these groups will interact with bioactive macromolecules depends mostly on the reactant and reaction conditions.[7] Therefore, HNE directly reacts mostly with proteins by binding to cysteine, histidine and lysine residues. It can also induce a sequence of complex biochemical reactions.[19,20] At high concentrations (100 μmol $dm^{-3}$ and more) HNE has acute cytotoxic effects that usually lead to cell death. Physiological concentrations of HNE, in tissues and in serum, are below or in the range of 1 μmol $dm^{-3}$. Although it is not certain yet which are the major biological activities of the aldehyde under physiological conditions, it seems that growth modifying activities may dominate.[21] Activities of adenylate-cyclase, guanylate-cyclase and phospholipase C are affected by HNE. Thus, HNE is involved in the regulation of cell growth, signal transmission and other important cell functions.[7] HNE is considered to be one of the major mediators of oxidative stress,[22] a chemotactic aldehyde end product of lipid peroxidation,[23] a major lipid peroxidation product,[24,25] and a causative factor of secondary tissue damage.[2,8,26]

In the first 25 years of its history, HNE was determined only by complex chemical methods (gas chromatography and mass spectroscopy).[25] In the 1990s, several polyclonal and monoclonal antibodies against HNE modified epitopes on proteins were developed, which resulted in more experimental studies.

According to the available data, there are 49 papers that use monoclonal antibodies against HNE conjugated with proteins, but the use of such antibodies coated with colloidal gold for immunoelectromicroscopy is very rare (only three publications).[27,28,29]

In only one study the authors evaluated the presence of HNE in inflammatory cells, but they used bacterial phagocytosis as a model and specific polyclonal antibodies recognizing 4-HNE-protein adducts and gp91-phox (flavocytochrome b large subunit) in cryofixed, molecular distillation-dried neutrophils.[27]

In our study, we used a modified immunogold method based on monoclonal antibodies for detection of HNE-histidine conjugates in formalin-fixed cells. In our experiment, we analyzed spleen tissue, peripheral blood leukocytes and peritoneal macrophages obtained from rats challenged i.p. by the application of tribomechanically micronized zeolites (MZ) that attract macrophages[30,31] acting against MZ as a foreign particle (unspecific immunogen).

Zeolites are hydrated natural and synthetic microporous crystals with structures containing $SiO_4$ and $AlO_4$ tetrahedral and linked by shared oxygen atoms.[32] Zeolites possess many biological properties as well as long-term chemical and biological stability.[33] After i.p. application of MZ, the number of peritoneal macrophages, as well as their production of superoxide anions, was seven times higher than in control mice.[34] However, NO generation was totally abolished. At the same time, translocation of p65 (NFkB subunit) to the nucleus of splenic cells was observed. There was no change in the concentration of liver TBARS (thiobarbituric acid-reactive substances) and spleen TSA (lipid-bound sialic acid), indicating that MZ does not have systemic pro-oxidative effects. This was important for our study aimed at verifying the specificity of the immunoelectronmicroscopy method of detecting intracellular HNE-histidine adducts.

## EXPERIMENTAL

### *Animals and Treatment*

Three months old male Wistar rats of an average weight of 300 g were used with water and food given *ad libitum*. The animals ($N$ = 7) were injected i.p. with 3 mg of MZ in 0.3 ml of Hank's solution and were kept individually in plastic cages under standard conditions (regulated air atmosphere, 12 hours day/night cycle) for 24 hours until they were sacrificed by ether. Rats were used for isolation of macrophages, leukocytes and spleen tissue.

Fine powder of natural clinoptilolites (MZ: micronized zeolite) from Slovakia was obtained by tribomechanical micronization.[31] Particle size analysis of the clinoptilolite showed that the maximum particle frequency appeared at 1 μm.

### *Processing of Cells and Tissues*

Macrophages, leukocytes and spleen tissue were collected under sterile conditions from each animal separately.

Macrophages were collected from the peritoneal cavity of rat 24 hours after i.p. administration of MZ (15 mg/rat). Cells were collected with sterile PBS (phosphate buffered saline, pH = 7.2), after mechanical provocation of the peritoneum. Macrophages were washed by centrifugation at 1100 rpm for 5 minutes at +4 °C and used for the analysis.

Leukocytes were isolated from blood taken from the heart with a heparinized syringe and placed into a heparin coated tube. Blood was allowed to settle for two hours; thus, the erythrocytes were on the bottom of the tube and leukocytes formed a white ring on the top of the erythro-

cyte pellet. Leukocytes from the top were collected, diluted in PBS and used for the analysis. According to the trypan blue exclusion assay, viability of the cells was >98 %.

Spleen tissue was isolated under sterile conditions, dissected into small pieces (<1 $mm^3$), left in PBS for 30 min and used in the experiment.

### *4-Hydroxynonenal Preparation*

The aldehyde, in the form of 4-hydroxy-2-nonenal-dimethylacetal (HNE-DMA) was kindly provided by the Institute of Molecular Biology, Biochemistry and Microbiology, Graz, Austria. Prior to the experiment, it was activated with 1 mmol $dm^{-3}$ HCl (Kemika, Croatia) for 1 hour. The concentration of HNE was spectrophotometrically determined (Biochrom 4060, LKB Pharmacia, USA). For the experiment, HNE was diluted in the DMEM medium (Dulbecco's Modified Eagle's Medium).

### *HNE Treatments* in vitro

Blood leukocytes and peritoneal macrophages ($7 \times 10^7$ in 2 ml), treated *in vitro* with 100 μmol $dm^{-3}$ HNE for 30 min at 37 °C, were taken as HNE-positive control samples. Very small spleen tissue pieces (1 $mm^3$) were also treated *in vitro* with 100 μmol $dm^{-3}$ HNE for 30 min at 37 °C, and were taken as HNE-positive control samples. The untreated samples were left, in the same condition, in plain DMEM medium. Afterwards, the samples were fixed in a 10 % buffered formalin solution for immunohistochemistry and immunocytochemistry, and in a mixture of glutaraldehyde/paraformaldehyde in cacodylate buffer for immunoelectronmicroscopy.

### *Immunohistochemical and Immunocytochemical Detection of HNE*

For immunostaining, slides were treated as described before, and specific monoclonal antibodies provided by Karl-Franzen's University in Graz, Institute of Molecular Biology, Biochemistry and Microbiology, Graz, Austria were applied.[17,35]

Monoclonal antibodies for the detection of HNE-modified proteins were obtained from the culture medium of the clone »HNE 1g4«, produced by a fusion of Sp2-Ag8 myeloma cells with B-cells of a BALBc mouse immunized with HNE modified keyhole limpet hemocyanine. The antibody is specific for the HNE-histidine epitope in HNE-protein (peptide) conjugates and gives only 5 % cross reactivity with HNE-lysine and 4 % with HNE-cysteine.[36]

For the immunocyto/histochemical detection of HNE-adducts, the immunoperoxidase (peroxidase labeled anti-peroxidase antibodies, PAP) technique was used, with secondary rabbit-anti-mouse antibodies (Dako, Denmark), applied on 5 μm sections of the formalin-fixed paraffin embedded sections of the samples. 3,3'-Diaminobenzidine tetrahydrochloride (DAB, Sigma, USA) was used as chromogen. The PAP-DAB staining indicated the presence of HNE as brown staining while no contrast-staining was used.

Specificity of anti-HNE immunostaining was routinely validated as described before[35,37] using HeLa cells pretreated with ranging concentrations of HNE (1–100 μmol $dm^{-3}$).

### *Immunoelectronmicroscopical Detection of HNE (Immunogold Method)*

Samples fixed in a mixture of glutaraldehyde/paraformaldehyde in cacodylate buffer (pH = 7.2) were first infiltrated with epoxy resin. Ultrathin sections (130 Å) were cut with a diamond knife on a Reichert ultramicrotom. Sections were mounted directly onto 200 mesh nickel grids. For immunostaining, endogenous peroxidase activity was blocked and osmium was washed off the sections with 3 % hydrogen peroxide in PBS, which makes the sections more hydrophilic. Murine monoclonal antibodies against HNE-histidine conjugates described before were used as the primary antibody diluted 1:10 in 1 % bovine serum albumin (BSA, Hamosan, Graz, Austria). The samples were incubated for 24 hours at +4 °C. The secondary antibody was a colloidal gold coated rabbit-anti-mouse immunoglobulin of 10 nm (Dako, Denmark) diluted in PBS (1:50). After immunolabeling, sections were counterstained with uranyl acetate (Agar, England) and Reynolds lead nitrate solution, and afterwards grids were analyzed by electron microscopy (Morgagni 268D, Philips).

## RESULTS

### *Analysis of HNE-histidine Adducts Distribution by Light Microscopy*

In control peripheral blood leukocytes, HNE positivity (determined by the PAP method) was hardly noticed and was present in only a few cells (Figure 1 $a_1$). It was mostly located adjacent to the cellular membranes. In contrast, in most of the peripheral blood leukocytes treated with 100 μmol $dm^{-3}$ HNE, very evident imunocytochemical HNE positivity was observed, mostly in cell membranes (Figure 1 $b_1$). Macrophages isolated from rat peritoneum showed results similar to blood leukocytes. HNE-untreated macrophages showed moderate HNE positivity near the cell membranes in some cells (Figure 1 $a_2$), while macrophages that were treated with 100 μmol $dm^{-3}$ HNE showed marked immunopositivity, particularly in the cell membrane region (Figure 1 $b_2$).

HNE-immunopositivity was seen in only some spleen cells in the tissue that was not treated with HNE (Figure 1 $a_3$), while *in vitro* treatment of spleen tissue with 100 μmol $dm^{-3}$ HNE resulted in remarkable immunopositivity (Figure 1 $b_3$).

### *Analysis of HNE-histidine Adducts Distribution by Electron Microscopy*

Ultrastructural analysis showed similar HNE-histidine adducts distribution in untreated blood leukocytes, peritoneal macrophages and spleen tissue cells. Samples that

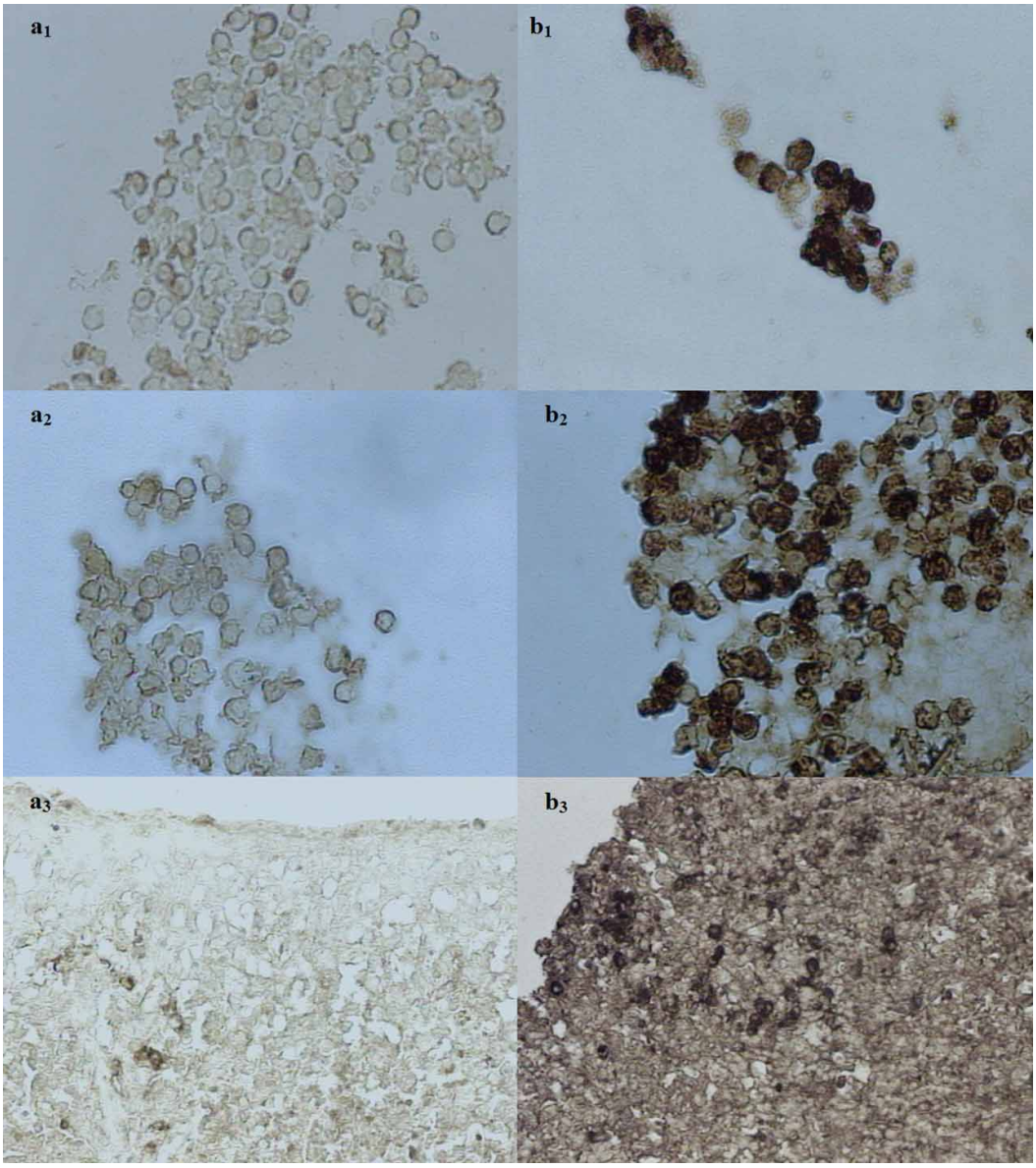


Figure 1. (a.) HNE positivity detection by the imunocytochemical PAP method in HNE-untreated samples; ($a_1$) leukocytes from blood (magnified 400 ×), ($a_2$) macrophages (magnified 400 ×), ($a_3$) spleen tissue (magnified 200 ×); (b.) HNE positivity detection by the imunocytochemical PAP method in samples treated with 100 μmol $dm^{-3}$ HNE; ($b_1$) leukocytes (magnified 400 ×), ($b_2$) macrophages (magnified 400 ×), ($b_3$) spleen tissue (magnified 200 ×).

were not treated with 100 μmol $dm^{-3}$ HNE had better preserved cell structures and moderate HNE positivity marked by 10-nm colloidal gold particles. HNE-histidine conjugates were detected inside their mitochondria, near phagosomes and membrane structures, beside macrophage granules or attached to the cellular membrane, but were rarely present in the cytosol or the nuclei (Figure 2 $a_1$, $a_2$, $a_3$). HNE-untreated leukocytes (Figure 2 $a_1$) had occasionally HNE positivity while membrane structures were intact. Macrophages that were not treated with HNE (Figure 2 $a_2$) had seldom HNE-histidine adducts in cytosol and in granules, which were of well preserved structure. Similarly, HNE-untreated spleen tissue (Figure 2 $a_3$) showed occasional presence of HNE-immunopositivity in cytosol and in the vicinity of the nucleus, with preserved integrity of the cellular organelles and the nucleus.

Ultrastructural analysis of leukocytes, macrophages and spleen tissues that were treated with 100 μmol $dm^{-3}$ HNE showed very evident HNE-positivity beside macrophage granules, near and on membrane structures and diffusely spread in the cytoplasm. Samples treated with 100 μmol $dm^{-3}$ HNE had slightly damaged cytoplasmic structures (Figure 2 $b_1$, $b_2$, $b_3$). HNE-treated samples of leukocytes (Figure 2 $b_1$) showed remarkable HNE-histidine positivity mostly on damaged membrane structures and in the vicinity of cellular membranes in the cytoplasm. Macrophages (Figure 2 $b_2$) treated with HNE showed HNE positivity around cytoplasmic granules, which were also

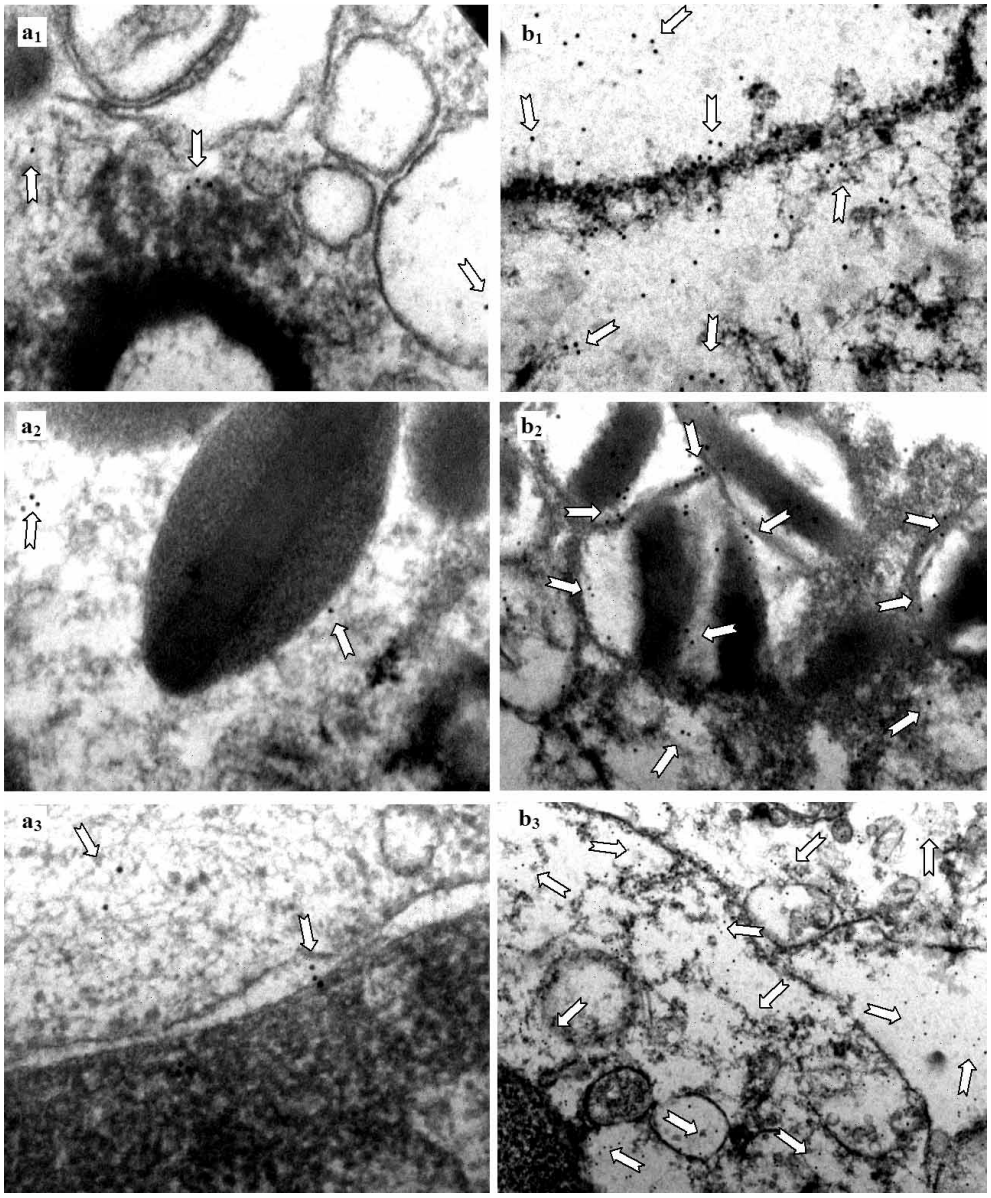


Figure 2. (a.) Electron microscopic analysis of HNE-untreated samples (marked by 10-nm colloidal gold particles): ($a_1$) peripheral blood leukocytes had occasionally HNE positivity while membrane structures were intact (magnified 89 000 ×), ($a_2$) peritoneal macrophages had seldom HNE-histidine adducts in cytosol and in granules, which were of well preserved structure (magnified 89 000 ×), ($a_3$) spleen tissue showed occasional presence of HNE immunopositivity in cytosol and in the vicinity of the nucleus, with preserved integrity of the cellular organelles and the nucleus (magnified 110 000 ×); (b.) Electron microscopical analysis of HNE positive control samples: ($b_1$) peripheral blood leukocytes showed remarkable HNE-histidine positivity mostly in damaged membrane structures and in the vicinity of cellular membranes in the cytoplasm (71 000 ×), ($b_2$) peritoneal macrophages showed HNE positivity around cytoplasmic granules, which were also damaged (magnified 71 000 ×), ($b_3$) spleen tissue showed very prominent positivity in cytosol and around damaged membrane structures and the nucleus (magnified 36 000 x).

damaged. Similarly, the spleen tissue (Figure 2 $b_3$) used as HNE-positive control showed very prominent positivity in cytosol and around damaged membrane structures and the nucleus.

## DISCUSSION

Since HNE is known as a second messenger of free radicals that interferes with cytokines, studying its role in inflammation is particularly interesting. The advantage of our immunodetection method, where genuine monoclonal antibodies raised against HNE-histidine adducts were used, is that biologically inactive HNE-glutathione conjugates are not recognized. Namely, HNE is mostly scavenged by binding to the cystein SH-group of glutathione (GSH), so there is no interaction with HNE-histidine conjugates analyzed.

It was observed that HNE-histidine conjugates could be detected by the standardized immunohistochemical PAP method, but the immunogold method was introduc-

ed because we wanted to determine, as far as possible, the exact location of HNE-histidine conjugates within the cells, *i.e.*, on particular cellular structures. HNE-histidine conjugates were clearly visible on electron microscopic images. These were apparently stable HNE-conjugates that were not washed out during mechanical and chemical treatments of cells and tissue samples, as it would be the case with HNE-GSH adducts.

HNE-histidine conjugates were occasionally detected in HNE-untreated macrophages, leukocytes and spleen tissue. Similar findings were also observed for various rat and human tissues (data not presented), indicating that this is not a histo- or species-specific finding and is not related to the MZ treatment *in vivo*. Since HNE-histidine conjugates were mostly found in phagosomes, mitochondria and cell membranes of leukocytes and macrophages, it is possible that these are the formation sites of HNE-protein adducts. This may also suggest the presence of higher amounts of particular HNE-binding proteins in these cellular organelles.

The highest onset of oxidative stress is usually extracellular or in peroxisomes where peroxidases and nonenzymatic processes of oxidative PUFA degradation cause HNE formation. Therefore, HNE positivities detected in the cells should correspond to the HNE-protein conjugates developed during oxidative burst. Interestingly, most of HNE positivity was localized at the membranes, even if the aldehyde was added exogenously (for positive controls), indicating a relatively high incidence of HNE-binding proteins with histidine within and near the membranes. This might be also relevant to the regulation of cellular activity under oxidative stress or oxidative burst. Namely, we used a relatively high concentration of HNE (100 μmol $dm^{-3}$ HNE) to get HNE-immunopositive staining of the cells, which we had previously found to increase the growth of lymphocytes *in vitro*, inhibiting the growth of leukemic cells in parallel.[38] In our current study, the same amount of aldehyde was associated with only moderate changes of the subcellular structures of leukocytes but with obvious immunopositivity of the HNE-histidine adducts.

As it is known that HNE can act as a chemoattractant,[23] we can assume that the production of HNE was not an accidental event, but an inflammation regulating one. We assume that MZ did not increase the HNE production in macrophages and lymphocytes from blood and spleen of MZ treated rats, although it strongly (seven fold) increased accumulation of macrophages in the peritoneum.[34] Namely, we found that MZ selectively reduced generation of HNE *in vivo* in tumor stroma (normal cells within the tumor tissue) after Doxorubicin treatment, leavin the onset of lipid peroxidation intact in malignant cells.[39] Therefore, the absence of HNE in the control, HNE-untreated cells was not surprising, irrespective of the treatment with MZ.

This is the first study where these particular antibodies specific for HNE-histidine conjugates were used in an immunogold method. The sensitivity of the immunogold method could be stronger if largely hydrophilic unicryl resin were used. The advantages of unicryl for staining and labeling lie both in its preservation of tissue structures and its sectioning characteristics, such that proteins, nucleic acids and macromolecules are presented at the surface of the sections for subsequent incubations.

A further goal of our research is to analyze biological effects of lipid peroxidation products using different methods, such as microarray evaluation of genome expression depending on the presence of HNE-histidine conjugates in inflammatory cells. This could be applied in pathological cells, such as leukemia and lymphoma cells, and central nervous system cells damaged by neurodegenerative diseases. Combined evaluation of biological effects of HNE and of subcellular distribution of HNE-protein adducts will increase our understanding of HNE as a signaling molecule and possibly as a major bioactive marker of oxidative stress.

*Acknowledgements.* – Financial support of the Croatian Ministry of Science, Education and Sports is gratefully acknowledged. The authors express their gratitude to Mrs. Gordana Strunjak for excellent technical support.

## ABBREVIATIONS

HNE, 4-hydroxy-2-nonenal
HNE-DMA, 4-hydroxy-2-nonenal-dimethyl-acetal
i.p., intraperitoneal
MZ, micronized zeolite
ROS, reactive oxygen species
PUFA, polyunsaturated fatty acids
PBS, phosphate buffered saline
DMEM, Dulbecco's Modified Eagle's Medium
PAP, peroxidase labeled anti-peroxidase
DAB, diaminobenzidine tetrahydrochloride
BSA, bovine serum albumin

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## SAŽETAK

### Nova metoda za dokazivanje HNE-histidinskih konjugata u štakorskim upalnim stanicama

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Oksidacijski stres, stanje prekomjernoga stvaranja reaktivnih kisikovih tvari, bitna je sastavnica različitih bolesti, ali i fizioloških procesa (upala). Često je razlika između fiziološkoga i patološkoga oksidacijskoga stresa pojava lipidne peroksidacije i njenih završnih toksičnih produkata, među kojima posebnu ulogu ima 4-hidroksi-2-nonenal (HNE), reaktivni aldehid koji tvori konjugate s bjelančevinama. Cilj ovoga istraživanja bio je utvrditi

distribuciju HNE-histidinskih konjugata u leukocitima tijekom nespecifične upalne reakcije. Rabili smo izvorna monoklonalna protutijela na HNE-histidinski konjugat, za imuno-citokemijsko, histokemijsko i elektronsko-mikroskopsko dokazivanje HNE-a u upalnim stanicama. Analizirano je tkivo slezene, leukociti iz krvi te makrofazi štakora kojima je intraperitonealno injiciran mikronizirani zeolit (MZ). HNE-histidinski konjugati pretežito su uočeni uz stanične membrane te pored fagosoma i makrofagnih granula. Imunodetekcija HNE-histidinskih konjugata mogla bi se stoga rabiti kao analitička imunokemijska metoda za istraživanja fizioloških i patoloških procesa te u patomorfološkim dijagnostičkim postupcima.