**APOPTOTIC CLEARANCE IN RABBITS WITH EXPERIMENTAL PULMONARY EMPHYSEMA**

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*In order to better understand pathogenesis of pulmonary emphysema, the model of experimentally induced pulmonary emphysema in Chinchilla rabbits was used for the estimation of apoptotic clearance of pulmonary tissue. Bronchoalveolar lavage was performed in three groups of animals: experimental group-E on hypercholesterolemic diet (4% edible oil solution of crystalline cholesterol), control group-$C_1$ on standard diet for that animal species and animals on oily diet-$C_2$. Apoptotic detection in cytocentrifuge preparations of lung washings was evaluated by in situ TUNEL. The property of alveolar macrophages to engulf apoptotic cells was estimated by light microscopy including 300 features (related subsequent steps: adsorption, internalization and intracellular processing of free apoptotic bodies) and was evaluated by scoring and indexing method. Internalization of apoptotic bodies by alveolar macrophages, as well as free apoptotic bodies were decreased in E compared to both $C_1$ and $C_2$ group (p<0.01 and p<0.05 respectively). Intracellular processing of apoptotic bodies by alveolar macrophages is significantly decreased in $C_2$ in comparison with E (p<0.05) and $C_1$ group (p<0.01). Apoptotic capacity of pulmonary tissue is significantly decreased in $C_2$ in comparison with $C_1$ group (p<0.01). The results implicate that immuno-metabolic competence of pulmonary tissue might be essentially associated with tissue remodeling in pulmonary emphysema.*

*Key words**: alveolar macrophages, apoptosis, clearance, experimental emphysema*

INTRODUCTION

Apoptosis is one of the crucial events in tissue remodeling which is included in tissue injury/repair processes (Uller *et al*., 2006). Apoptotic cell death is an important pathophysiological mechanism in many diseases (Savill and Fadok, 2000; Fadok *et al*., 2001).

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Neutrophil apoptosis represents a major mechanism involved in the resolution of inflammation (Hannah *et al*., 1995). Neutrophils are programmed to undergo constitutive apoptosis. This process locks the cell into a non-secretory mode and initiates rapid engulfment and removal by inflammatory macrophages as key determinants affecting the resolution of airway inflammation (Brazil *et al*., 2005).

Clearance of apoptotic cells by phagocytes is a complex and incompletely understood process that involves both recognition and ingestion steps (Schlegel *et al*., 1996). Clearance of free apoptotic bodies (FAB) – cells of monocyte-granulocyte lineages by alveolar macrophages is essential in maintaining the lungs tissue architecture.

Although we can not understand the precise physical process guided by cell to cell signaling malfunctioning ingestion of apoptotic cells may induce alveolar macrophages to produce proinflammatory cytokines as reported Hu *et al*., 2000. This mechanism could be responsible for the devastating effect on pulmonary tissue *in situ*. The inflammatory response in pulmonary emphysema typically shows evidence of the activation of innate and acquired inflammatory processes. The accumulation of these inflammatory components contributes to lung injury in these patients and serves as a self-perpetuating stimulus for further immune activation (Sharafkhaneh *et al*., 2008).

Apoptotic capacity (AC) of alveolar macrophages to clear pulmonary tissue from free apoptotic bodies, excluding macrophages undergoing apoptosis themselves, was estimated in order to better understand pathological physiology of pulmonary emphysema.

## MATERIAL AND METHODS

*Experimental animals*
In this study Chinchilla rabbits (Military farm, Torlak, Belgrade, Serbia), of both sexes, body weight 1300-2500 g at the beginning of the experiment were used. The animals were kept under the same conditions (room temperature 16-18ºC), had access to standard rabbit chow (Veterinary Institute, Zemun, Serbia) and tap water *ad libitum*.

All experimental procedures were performed in accordance with the European Council Directive (86/609/EEC), and were approved by the Animal Care Committee of the University of Belgrade

*Experimental model of pulmonary emphysema*
Experimental atherosclerosis and consequent experimental pulmonary emphysema were induced on rabbits by diet rich in cholesterol (4% crystalline cholesterol in eatable oil solution, *per os*, 6 mL daily, for two months).

Investigated animals were divided into three groups:

1. Control group $C_1$ (n=10) – animals on non-supplemented standard diet ,

2. Experimental group E (n=13) – animals on hypercholesterolemic diet (4% crystalline cholesterol (ICN Galenika, Serbia) in edible oil ("Dijamant", Subotica, Serbia) solution, *per os*, 6 mL daily, for two months), and

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3. Oil control group $C_2$ (n=7) – animals on oily diet (eatable oil "Dijamant", Subotica, Serbia) solution, *per os*, 6 mL daily, for two months.

The animals were sacrificed by intracardial air injection and bronchoalveolar lavage was performed immediately.

*Bronchoalveolar lavage*

Bronchoalveolar lavage (BAL) was performed using Phegel's method, modified by Schuyler and Todd (1981). One mL saline, prewarmed at 37°C, was successively instilled and reaspirated using an intratracheal plastic cannula. The procedure was repeated ten times. BAL specimens were immediatly transfered to the laboratory on ice where BAL recovery, BAL cell viability, BAL cell counting, cytocentrifuge slide preparations for lavage cell differential counting and cytochemical analysis were performed. After centrifugation (300 g, 5 min, 4°C), pelleted cells were resuspended in Haemacel (Jugoremedia) solution and cytocentrifuge preparations were prepared by transferring 100 μL (1.5 x $10^6$/mL cells) into cytocentrifuge (Roto Silenta/RP Hettich). The preparations were spun at 450 r.p.m. for 10 min and a set of cytocentrifuge slide preparations air dried at room temperature. From each lavage a sample for cytochemical staining was made.

Pulmonary tissue (right low lobus) samples stained with hematoxylin-eosin (HE) was taken for pathohistological analysis using a light microscope (Opton Photomikroskop III).

*TUNEL assay*

The study of apoptosis was designed regarding cytochemical indexing and scoring as originally described by Zunic *et al*. (2004).

Apoptotic detection in cytocentrifuge preparations of BAL cell suspensions was evaluated by light microscopy using TUNEL *in situ* cytochemical method (Boehringer Mannheim, In Situ Cell Death Detection Kit, POD; Cat. No: 1 684 817) and modified for BAL cytocentrifuge preparations (Zunic *et al*., 2004). In order to destroy endogenous peroxidase, selected cytocentrifuge BAL preparations were incubated with blocking solution (0.3% $H_2O_2$ in methanol) for 30 min at room temperature. After that, the slides were rinsed in PBS (pH 7.4), immersed in permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 15 min at room temperature and rinsed twice in PBS for 5 min. The samples treated with DNase I (100 μg/mL) for 15min at room temperature prior to labeling, served as positive controls. The slides were labeled for 1 h at 37°C in a dark, humidified chamber. The labeling solution (50 μL) contained calf thymus TdT (terminal deoxynucleotidyl transferase) and a nucleotide mixture in the reaction buffer with modified nucleotides (fluorescein-dUTPs). The enzymatic reaction was terminated by immersing the slides in 3.0 M sodium chloride, and 30 mM sodium citrate solution (TB) for 15 min at room temperature. The slides were washed twice in PBS (pH 7.4), covered with 2% aqueous bovine serum albumin solution for 10 min at room temperature, rinsed in PBS and incubated with 50 μL peroxidase-conjugated anti-fluorescein antibodies (Converter-POD) in a dark, humidified chamber for 30-60 min at 37°C. After three rinses with PBS, the samples were

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stained with diaminobenzidine (DAB) solution (6 mg in 10 mL Tris-HCl, pH 7.6) containing 0.03% $H_2O_2$, for about 10 min at room temperature. Finally, the sections were lightly counterstained with Harris' haematoxylin, dehydrated in alcohol and cleared in xylene. The slides were mounted with Permount (Fisher Chemical) and analyzed under a light microscope (ZEISS, Axioplan) using an immersion objective. Figure 1 is a schematic representation of the unique features from photomicrographs of BAL cytocentrifuge preparations stained by TUNEL.

Indexing and scoring was performed and calculation of apoptotic capacity (AC) made as described by method of Zunic *et al*. (2004). Adopted for the present study, AC was estimated by light microscopy including 300 features per sample. These features are related to subsequent steps (adsorption, internalization and digestion of apoptotic bodies by alveolar macrophages).
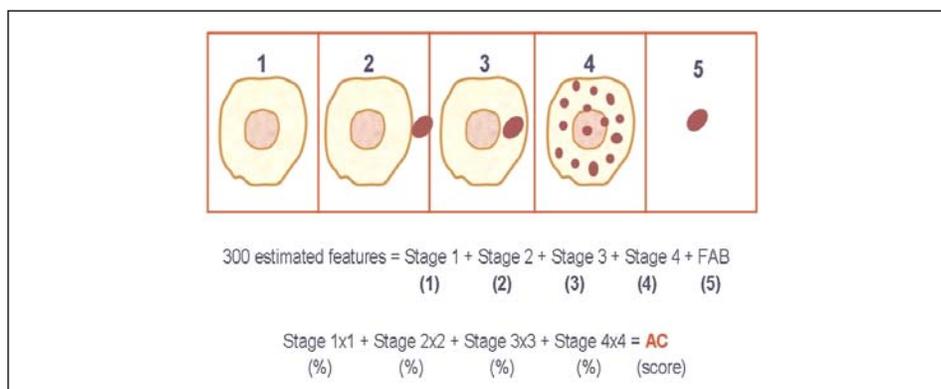


Figure 1. Schematic presentation of cells of interest in the evaluation of apoptotic capacity of pulmonary tissue
Stage 1 – clear alveolar macrophages;
Stage 2 – apoptotic bodies adherent to alveolar macrophages;
Stage 3 – apoptotic bodies internalized by alveolar macrophages;
Stage 4 – apoptotic bodies digested inside the alveolar macrophages;
Stage 5 – free apoptotic bodies;
AC – apoptotic capacity

Small dark contours are free apoptotic bodies (FAB). Big white nucleated cells represent AM in four features (1-4) important for the calculation: clear AM (1); apoptotic bodies adherent to AM (2); apoptotic bodies internalized by AM (3); apoptotic bodies digested inside the AM (4) and FAB (5).

Each feature was expressed as a percentage of the sum: total macrophages (stages 1-4) + free apoptotic bodies = 300 features.

Apoptotic capacity (AC) - relation between presence of FAB and their removal by AM was calculated as a sum of multiples of the corresponding stage (their relative percents) and weighting factors.

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*Statistical analysis*

Data were expressed as mean ± SD. Statistical analysis was performed with Student's t test for two samples. Statistical analysis of the results was performed using STATISTICA for Windows program, suitable at PC.

## RESULTS

Results are presented in Figures 2-4 and Tables 1 and 2.

There were well pronounced emphysematous changes in pulmonary tissue in the group on hypercholesterolemic diet (E) (Figure 4). In the group of animals on oil diet ($C_2$ group) there were some destructive alveolar changes and inflammatory reactions in the pulmonary tissue (Figure 3) in comparison with the normal histological appearance of pulmonary tissue ($C_1$ group) (Figure 2).
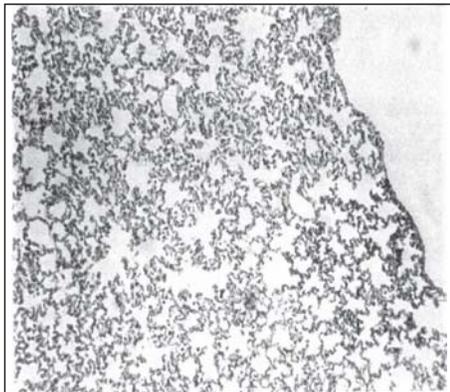


Figure 2. Photomicrography of a
histological section of pulmonary
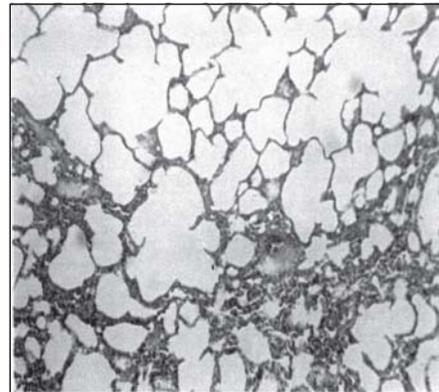tissue of an animal in $C_1$ group
(HE x 80)



Figure 3. Photomicrography of a
histological section of pulmonary
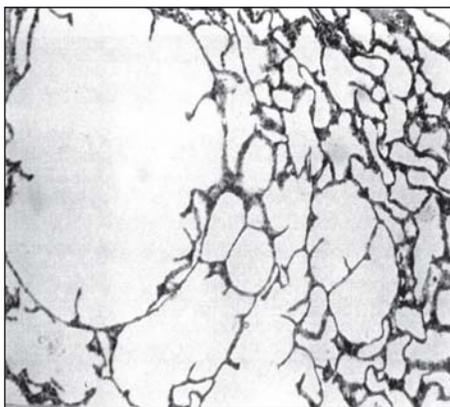tissue of an animal in $C_2$ group
(HE x 80)



Apoptotic capacity of alveolar macrophages (AM) to engulf apoptotic bodies was slightly decreased in animals on a hypercholesterolemic diet and significanly less in animals on oil suplemented diet (123.14±22.04) in comparison with the control group (154.14±31.63) (p<0.01).

Figure 4. Photomicrography of a histological
section of pulmonary tissue of an
animal in E group (HE x 80)

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Internalization of apoptotic bodies is decreased in animals on hypercholesterolemic diet ($2.36 \pm 0.81$) compared to both $C_1$ ($4.00 \pm 1.25$) ($p < 0.01$) and $C_2$ group ($4.42 \pm 1.51$) ($p < 0.05$). The intracellular processing of apoptotic remnants is significantly decreased in animals on the oil diet ($11.00 \pm 2.94$) in comparison with the experimental ($16.23 \pm 5.28$) ($p < 0.05$) and control group ($17.87 \pm 5.59$), $p < 0.05$ and $p < 0.01$ respectively. Relative percents of free apoptotic bodies are significantly decreased in experimental animals ($17.50 \pm 6.16$) compared to both control animals ($35.67 \pm 11.06$) and animals on the oil supplemented diet ($29.57 \pm 9.98$), $p < 0.01$ and $p < 0.05$ respectively (Table 1 and Table 2).

Table 1. Relative percents of stages of apoptotic clearance by AM in investigated groups

| FEATURES | GROUPS | | |
|---|---|---|---|
| | $C_1$ (n=10) (mean±SD) | $C_2$ (n=70 (mean±SD) | E (n=13) (mean±SD) |
| 1 – Clear AM | 44.57±13.51 | 41.57±13.70 | 39.92±13.55 |
| 2 – AB attached to AM | 12.50± 4.13 | 12.89± 3.44 | 10.54± 3.62 |
| 3 – AB internalizad by AM | 4.00± 1.25 | 4.42± 1.51 | 2.36± 0.81 |
| 4 – AB processed in AM | 17.87± 5.59 | 11.00± 2.94 | 16.23± 5.28 |
| 5 – FAB | 35.67±11.06 | 29.57± 9.98 | 17.50± 6.16 |
| AC | 154.14±31.63 | 123.14±22.04 | 145.56±46.44 |

AM – alveolar macrophages; AB – apoptotic bodies; FAB – free apoptotic bodies; AC – apoptotic capacity

Table 2. Statistical significance of differences for apoptotic parameters between investigated groups

| FEATURES | GROUPS        COMPARED | | |
|---|---|---|---|
| | $C_1/C_2$ | $E/C_1$ | $E/C_2$ |
| 1 – Clear AM | ns | ns | ns |
| 2 – AB attached to AM | ns | ns | ns |
| 3 – AB internalizad by AM | ns | $p < 0.01$ | $p < 0.05$ |
| 4 – AB processed in AM | $p < 0.01$ | ns | $p < 0.05$ |
| 5 – FAB | ns | $p < 0.01$ | $p < 0.05$ |
| AC | $p < 0.01$ | ns | ns |

AM – alveolar macrophages; AB – apoptotic bodies; FAB – free apoptotic bodies; AC – apoptotic capacity; ns – non significant

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## DISCUSSION

In the present study, level of apoptosis of cells of granulocyte-monocyte lineage was observed and evaluated in terms of clearance of apoptotic cells by alveolar macrophages.

Apoptotic capacity of pulmonary tissue of rabbits reflects the relation between the presence of free apoptotic bodies originated from cells of the granulocyte lineage in lung washings, and their removal by phagocytosis by non-apoptotic alveolar macrophages.

Results of the present study show that the apoptotic clearance by alveolar macrophages was significantly decreased in the oil-fed group in comparison with control animals. Regarding findings of Baudry *et al.*, 2001 it is likely that variations of the amount of one component (execs oil diet) can induce rearrangements of the membrane. Report of Elias and Lee 2005 support an idea that changes of membrane micro-architecture may lead to a change of regulatory mechanisms in the lipid metabolism, including ceramide. Lipid mediator ceramide contributes to tissue destruction in pulmonary emphysema by promoting apoptosis of structural cells in the lung. Deregulation of ceramide biosynthesis is coexisting with changes in cholesterol derivatives (Storey *et al*., 1998). Moreover, ceramide was proposed as a potent inducer of apoptosis of structural cells in the lungs (Elias and Lee, 2005). Reliance on apoptosis and lipid mediators may be helpful in understanding the pathways that lead to alveolar destruction and pulmonary emphysema. Some types of lipids in the cell membranes, associated with detergent-resistant lipid domains or "rafts", contain cholesterol and ceramide molecules. A property of the cholesterol molecule is that it may help to stabilize boundaries between coexisting lipid domains (Maxfield and Tabas, 2005). In animals on hypercholestrolaemic diet, cholesterol might be responsible for modulation of membrane fluidity. Peddada *et al*., 1997 and Haines, 2001 discussed the changed properties of phospholipid bilayer. These changes might be partly included in changed phagocytic ability of AM.

Recognition of apoptotic clearance might be represented by adhesion of apoptotic bodies to alveolar macrophages. In our study there is no statistically significant difference in the adhesion of apoptotic cells to alveolar macrophages between investigated groups, but internalization of free apoptotic bodies was significantly decreased in rabbits with experimental emphysema. During the process of engulfing apoptotic cells, AM up-regulate the key lipid transporter, this leads to enhanced cholesterol efflux from the phagocytes (Gerbod-Giannone *et al*., 2006). Our results showed a decrease of FAB in BAL specimens in group of animals treated with oil + cholesterol diet. There is a possibility that in animals on hypercholesterolemic diet in the present study the significant decrease of FAB is due to action of pro-death messenger (Rossi and Gaidano, 2003). This is in contrast with findings of Henson and Tuder (2008) that increased levels of apoptotic cells in the sputum and lung tissue is a peculiarity of several serious respiratory diseases, including chronic obstructive pulmonary disease (COPD).

Impaired apoptotic clearance by AM in animals with oil overload is not in relation with the extent of alveolar wall destructive changes. Lipid handling by

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macrophages plays an important role in atherosclerosis, and so it is interesting that there is considerable overlap in the cellular mechanisms that regulate lipid metabolism and apoptotic cell engulfment (Elliott and Ravichandran, 2010). Possible therapeutic challenges could be proposed, starting from antibiotics and statines (Krysko and Vandenabeele, 2010). Opinion of Walsh (2007) argues toward potential protective effects of statins on the enhancement of phagocytosis of apoptotic granulocytes by macrophages.

## CONCLUSIONS

The present study showed that in rabbits with experimental pulmonary emphysema internalization of apoptotic bodies by alveolar macrophages, as well as intracellular processing of apoptotic bodies might be significantly influenced by cholesterol (+ oil) reach diet.

The precise knowledge of cell mechanisms leading to decreased clearance of apoptotic cells in COPD, including pulmonary emphysema is limited. It is likely that further research could reveal numerous overleaping molecules leading to better understanding of pathogenic mechanisms in COPD and particularly, pulmonary emphysema. Special challenge in this strategy is to understand pathophysiological mechanisms for impaired apoptotic cell clearance which is the subject of the present study, as well as experimental evidences for possible protective drug and diet effects.

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## APOPTOTSKI KAPACITET U KUNIĆA SA EKSPERIMENTALNIM EMFIZEMOM PLUĆA

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### SADRŽAJ

U cilju boljeg razumevanja patogeneze plućnog emfizema, u radu je korišćen eksperimentalni model emfizema pluća na činčila kunićima za procenu apoptotskog kapaciteta plućnoga tkiva. Bronholaveolarna lavaža je urađena na tri grupe životinja: eksperimentalnoj grupi-E na hiperholesterolskoj dijeti (4% uljani

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rastvor kristalnog holesterola), kontrolnoj grupi-$C_1$ na standardnoj dijeti za tu životinjsku vrstu i grupi životinja na uljanoj dijeti-$C_2$. Određivanje apoptotskih parametara cito-centrifužnih preparata bronhoalveolarnog lavata vršeno je posle bojenja preparata TUNEL *in situ* citohemijskim metodom. Sposobnost alveolarnih makrofaga da odstrane apoptotske ćelije fagocitozom procenjivana je svetlosnom mikroskopijom na 300 prikaza po preparatu (prikazi uključuju: adsorpciju, internalizaciju i intracelularno procesiranje apoptotskih tela) i evaluirana metodom indeksiranja i skora. Internalizacija apoptotskih tela alveolarnim makrofazima, kao i relativni procenat slobodnih apoptotskih tela, bili su signifikantno smanjeni u E grupi poredeći sa $C_1$ ($p < 0.01$) i $C_2$ grupom ($p < 0.05$). Intracelularno procesiranje apoptotskih tela alveolarnim makrofazima bilo je signifikantno smanjeno u $C_2$ u odnosu na E ($p < 0.05$) i $C_1$ grupu ($p < 0.01$). Apoptotski kapacitet tkiva pluća bio je signifikantno smanjen u $C_2$ u poređenju sa $C_1$ grupom ($p < 0.01$). Ovi rezultati ukazuju da imuno-metabolička kompetentnost plućnog tkiva može biti suštinski povezana sa remodelovanjem tkiva pluća u eksperimentalnom emfizemu pluća.