

## HIGHER APOPTOTIC CELL RATE IN BALKAN ENDEMIC NEPHROPATHY – STEREOLOGIC ANALYSIS

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**SUMMARY** – The aim of this study was to correlate apoptotic cell rate from different nephron segments between control group and groups of patients with Balkan endemic nephropathy (BEN). Kidney specimens of 20 patients with clinically and epidemiologically confirmed BEN were compared with biopsy material of 10 patients (group I, non BEN) without glomerular or tubulointerstitial disease. Out of 20 patients with BEN, 10 suffered and died from BEN (group II, BEN) and 10 patients (group III, BEN/CV) suffered from BEN but died from cardiovascular disease. Patient age ranged from 40 to 50 years. The apoptotic cell rate was measured in proximal and distal tubules and in collecting ducts using the 40X objective with a calibrated eyepiece multipurpose M 42 test system according to Weibel. Comparison of all three nephron segments yielded statistically significant differences in volume density of apoptotic cells in proximal tubules and in collecting ducts among all three patient groups (non BEN *vs.* BEN, non BEN *vs.* BEN/CV and BEN *vs.* BEN/CV,  $P < 0.001$  all). Statistically significant difference in apoptotic cell rate was also found in distal tubules between non BEN and BEN groups and non BEN and BEN/CV groups, but not between BEN and BEN/CV groups. Our results showed a statistically significant increase of apoptotic cells in all three nephron segments in patients with BEN (BEN and BEN/CV) compared to control group. The highest number of apoptotic cells was found in distal tubules in the groups of patients with BEN and BEN with coexisting cardiovascular disease, suggesting that these cells might be most frequently and most severely injured in patients with BEN.

*Key words: Balkan nephropathy; Apoptosis; Nephron; Stereology*

### Introduction

Balkan endemic nephropathy (BEN) is a slowly progressive, chronic tubulointerstitial disease of still not fully understood etiology, which leads to terminal renal failure at the age of 40-50<sup>1,2</sup>. It appears in endemic areas of Croatia, Serbia, Bosnia, Bulgaria and

Romania. In Croatia, BEN is restricted to the area of Brodska Posavina (a geographically compact area bounded by the Sava river in the south and by Slavonski Brod in the west), where the prevalence of BEN reached 4.4% of the population in affected villages<sup>3</sup>.

Since the 1950s, when BEN was initially described, there have been several proposed criteria for its diagnosis, with WHO criteria being mostly used<sup>4</sup>. The International Panel of BEN Investigators has recently suggested unified criteria: (1) epidemiologic criteria; (2) demonstration of the glomerular filtration rate decrease, proteinuria  $< 1$  g/24 h, microalbuminuria,

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tubular markers (glucosuria, increased urinary excretion of  $\beta_2$ -microglobulin or  $\alpha_1$ -microglobulin), typical renal histology; and (3) exclusion of other known kidney diseases<sup>5</sup>.

A higher prevalence of urothelial tumors of renal pelvis and ureter has been described in the inhabitants of endemic areas<sup>6,7</sup>. Investigations concerning the etiology of BEN suggest that it is probably an environmentally induced disease. Although family clustering is one of epidemiological characteristics of BEN, hereditary hypotheses have not been confirmed<sup>8,9</sup>. Ochratoxin A (OTA) and aristolochic acid (AA) are the causative agents most frequently considered in BEN etiology.

OTA is a mycotoxin produced by the fungal species *Aspergillus* and *Penicillium*, which has been found in blood samples from inhabitants of endemic villages (revealing their frequent exposure to this toxin)<sup>10,11</sup>.

Although a potent nephrotoxin and renal carcinogen, according to recent studies, OTA is not likely to be an etiologic factor involved in BEN because human exposure is several orders of magnitude below doses known to cause nephrotoxicity and tumor formation in laboratory animals<sup>12</sup>.

Aristolochic acid, found in herbs used in Chinese medicine, has been reported as an etiologic factor of a rapidly progressive tubulointerstitial nephritis with histologic characteristics similar to BEN<sup>13,14</sup>. Recent studies show that AA can induce proximal tubule apoptosis and epithelial to mesenchymal transformation<sup>15</sup>.

Histopathologic findings of the kidney in BEN highly depend on the stage of the disease. In the early stages of the disease, kidneys are of normal size and weight, with an increased amount of interstitial light eosinophilic acellular material through the cortex and smaller amount in the cortico-medullary junction. The epithelium of the compressed cortical, mostly proximal tubules shows different degenerative changes with an increased number of mitotic figures. In chronic cases, kidneys are very small with diffuse cortical interstitial fibrosis decreasing from the outer to inner cortex. A large number of tubules disappear, with frequent degenerative or necrotic changes of their epithelial cells. Reduction of the lumen and obliteration of small and middle arteries is a constant finding<sup>16</sup>.

Apoptosis is a type of cell death that occurs after activation of an internally controlled suicide program.

It is present in some physiologic processes such as embryogenesis or development of neural system, but is also seen in a variety of pathologic states (after toxic or irradiation cell injury)<sup>17</sup>. At the molecular level, apoptosis is induced by activation of the caspase system<sup>18</sup>. Protein p53 and Bcl-2 family member proteins, including the anti-apoptotic Bcl-2 protein and the pro-apoptotic Bax protein, are important regulators of apoptosis.

The aim of the study was to correlate apoptotic rate in different nephron segments between non BEN control group and groups of BEN patients.

## Material and Methods

We analyzed kidney specimens from three groups of 10 patients aged 40-50. Group I (non BEN) consisting of 10 patients that had never lived in the endemic area and had no signs of glomerular or tubulointerstitial disease served as control group. The majority of these patients were from Zagreb, Croatia. Group II (BEN) included 10 patients having suffered and died from BEN, whose disease was clinically and epidemiologically confirmed. Group III (BEN/CV) included 10 patients who suffered from BEN but died from cardiovascular disease (heart attack, chronic heart failure). Patients with BEN were from endemic villages in Croatia (Brodsko Posavina).

Specimens from patients with BEN represented archival material isolated approximately 8-10 hours after death, fixed in 10% buffered formalin, paraffin embedded, cut at 5  $\mu$ m and routinely stained with hematoxylin and eosin, while specimens from control group (non BEN) were surgical materials prepared and stained in the same manner.

On histologic examination, in tissues stained with hematoxylin and eosin apoptotic cell was identified as a round or oval mass of intensely eosinophilic cytoplasm with dense nuclear chromatin fragments, after fragmentation undergoing to apoptotic bodies composed of cytoplasm and tightly packed organelles with or without nuclear fragments (Fig. 1).

Apoptotic cells and apoptotic bodies were counted in proximal and distal tubules and in collecting ducts using the 40X objective of light microscope with a calibrated eyepiece multipurpose M 42 test system according to Weibel<sup>19</sup>. The apoptosis volume density was estimated by point counting as follows:

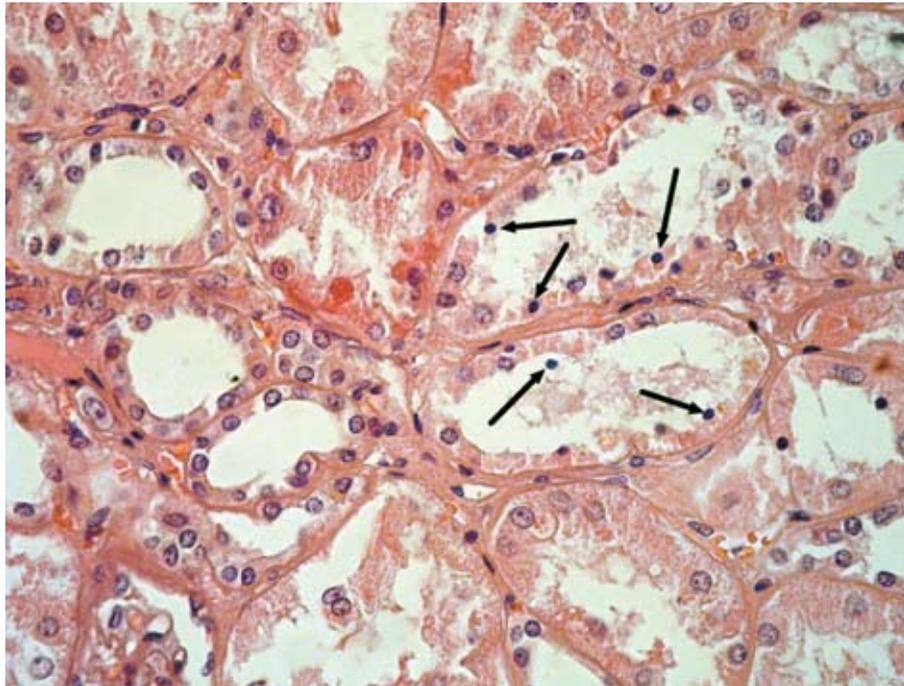


Fig. 1. Apoptotic cells (marked with black arrows) in distal tubules of kidney from a patient with BEN (HE  $\times$  400).

$V_v = A/Pt$ , where  $V_v$  represents volume density, A represents summation of the points falling on the apoptotic cells and apoptotic bodies, and Pt represents summation of the points falling on apoptotic cells, apoptotic bodies and epithelial cells.

Statistical analysis was done using ANOVA post hoc Scheffe test. The level of significance was set at  $P < 0.001$ .

## Results

Patients without BEN (group I) had the lowest number of apoptotic cells in all three nephron segments, compared to groups of patients with BEN (groups II and III). In group II (BEN), apoptotic rate

was highest in distal tubules, while comparison of all three groups of patients showed highest apoptotic rate in group III (BEN/CV), especially in distal tubules and collecting ducts. The range and mean values of volume density of apoptosis, representing its incidence in different nephron segments in all three patient groups, are shown in Table 1.

Comparison of all three nephron segments in all patient groups yielded statistically significant differences in volume density of apoptotic cells in proximal tubules among all three groups (non BEN *vs.* BEN, non BEN *vs.* BEN/CV and BEN *vs.* BEN/CV,  $P < 0.001$  all). Statistically significant between-group difference in apoptotic rate was also found in distal tubules between groups I (non BEN) and II (BEN)

Table 1. Maximal, minimal and mean volume density in different nephron segments in patients without BEN, with BEN and with coexisting BEN and cardiovascular disease

	Proximal tubules	Distal tubules	Collecting ducts
Non BEN	0.031-0.121 (mean 0.065)	0.021-0.112 (mean 0.065)	0.011-0.103 (mean 0.056)
BEN	0.085-0.153 (mean 0.114)	0.136-0.263 (mean 0.212)	0.071-0.186 (mean 0.130)
BEN/CV	0.090-0.221 (mean 0.164)	0.202-0.357 (mean 0.261)	0.096-0.363 (mean 0.246)

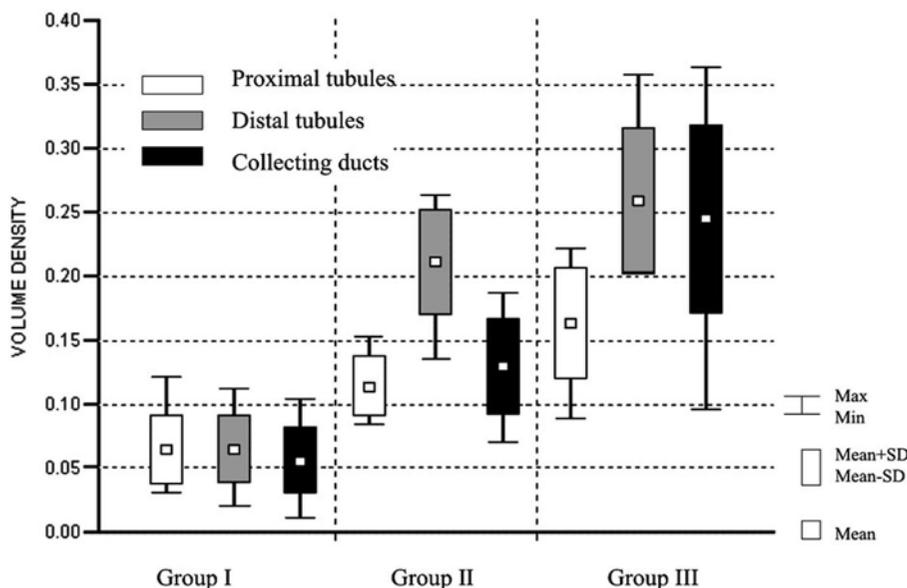


Fig. 2. Box plots with volume density of apoptotic cells in proximal and distal tubules and collecting ducts from all three groups of patients.

and between groups I (non BEN) and III (BEN/CV), but not between groups II (BEN) and III (BEN/CV). In collecting ducts, statistically significant difference was found among all three patient groups. Results are graphically presented with box plots in Figure 2.

## Discussion

Our study showed a statistically significant increase of apoptotic cells in all three nephron segments in patients with BEN and those with BEN and coexisting cardiovascular disease as compared to control group. The group of patients with BEN and cardiovascular disease (group III) had a significantly higher incidence of apoptotic bodies in proximal tubules and collecting ducts in comparison to the group of patients with BEN (group II), while no statistically significant difference between these two groups was recorded for distal tubules.

It is well known that BEN is chronic tubulointerstitial nephritis of the still not fully understood etiology and exact pathogenesis. Morphologically, the kidneys of BEN patients are very small with an end stage weight of 20–40 grams, and histologically, there is global sclerosis sparing juxtaglomerular nephrons and tubulointerstitial nephritis characterized by atrophy and collapsing tubules.

Koseki *et al.* were the first to implicate an important role of apoptosis in kidney embryogenesis, while Wiegele *et al.* noticed the higher number of apoptotic cells in proximal tubules in the early stage of ischemia and in distal tubules in conditions with long lasting ischemia<sup>20,21</sup>. It is obvious that in the advanced stages of endemic nephropathy, there is also significant ischemia caused by the reduction of blood flow and loss of interstitial vasculature. This was probably the cause of higher apoptotic rate in distal tubules of kidney in our patients with endemic nephropathy. In experimental works on rat kidneys, Thomas *et al.* observed that the number of apoptotic cells significantly increased with the intensity of glomerular sclerosis and tubulointerstitial fibrosis<sup>22</sup>. Tubular apoptosis as well as interstitial apoptosis plays an important role in decrease of kidney size in obstructive kidney disease<sup>23</sup>.

Schwerdt *et al.* investigated the role of mitochondria in OTA induced apoptosis of renal collecting duct derived MDCK-C7 cells, and showed that OTA may increase caspase activity in human proximal tubular cells in culture and that OTA induced apoptosis had a more damaging effect in acidified environment than in alkaline conditions<sup>24</sup>.

In a study by Pozdzik *et al.*, administration of AA to rats led to progressive tubular atrophy, which was related to defective regeneration of proximal tubular

epithelial cells and induction of apoptosis secondary to caspase-3 activation<sup>15</sup>. Chen *et al.* suggest that down-regulation of DNA repair gene expression in proximal tubular cells may be the mechanism responsible for AA-induced genotoxicity. They also observed down-regulation of the gene expression of anti-oxidant enzymes<sup>25</sup>.

In our pilot study, apoptosis was observed in different segments of nephron in patients with BEN<sup>26</sup>. Although some authors have pointed out the significance of apoptosis in the pathogenesis of BEN<sup>27,28</sup>, we found no studies correlating apoptotic cell rate between different segments of nephron.

The high number of apoptotic cells found in distal tubules in the groups of patients with BEN (group II) and BEN with coexisting cardiovascular disease (group III) without significant difference in distal tubules between these two groups could implicate that these cells are most frequently and most severely injured in patients with BEN; however, the true meaning of these findings should be additionally analyzed.

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### Sažetak

#### POVIŠENA GUSTOĆA APOPTOTIČNIH STANICA U BALKANSKOJ ENDEMSKOJ NEFROPATIJU – STEREOLOŠKA ANALIZA

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Cilj ove studije bio je usporediti gustoću apoptotičnih stanica u različitim dijelovima nefrona između kontrolne skupine i skupine bolesnika oboljelih od balkanske endemske nefropatije (BEN). Uzorci tkiva bubrega 20 bolesnika u kojih je klinički i epidemiološki dokazan BEN uspoređeni su s biopsijskim materijalom 10 bolesnika bez glomerularne ili tubulointersticijske bolesti bubrega (skupina I, non BEN). Od 20 bolesnika oboljelih od BEN 10 je umrlo zbog te bolesti (skupina II, BEN), dok je preostalih 10 bolovalo od BEN, ali je uzrok smrti bila istodobno postojeća kardiovaskularna bolest (skupina III, BEN/CV). Dob bolesnika bila je od 40 do 50 godina. Količina apoptotičnih stanica mjerena je u proksimalnim i distalnim tubulima te u sabirnim kanalicima, pod povećanjem od 40x primjenom testnog sustava M 42 s kalibriranim okularom prema Weibelu. Uspoređujući sva tri segmenta nefrona utvrđena je statistički značajna razlika u gustoći apoptotičnih stanica između stanica proksimalnih tubula i sabirnih kanalića u sve tri skupine bolesnika (non BEN *vs.* BEN, non BEN *vs.* BEN/CV i BEN *vs.* BEN/CV,  $P < 0,001$  svi). Statistički značajna razlika u gustoći apoptotičnih stanica uočena je i u distalnim tubulima između skupina non BEN i BEN te non BEN i BEN/CV, ali ne među skupinama BEN i BEN/CV. Rezultati su pokazali statistički značajnu razliku u gustoći apoptotičnih stanica u sva tri segmenta nefrona u bolesnika oboljelih od BEN (BEN, BEN/CV) u usporedbi s kontrolnom skupinom. Najveći broj apoptotičnih stanica pronađen je u distalnim tubulima bolesnika s BEN te onih s BEN i istodobnom kardiovaskularnom bolešću. Dobiveni rezultati upućuju na to da su u bolesnika s BEN stanice distalnih tubula vjerojatno najčešće i najjače zahvaćene.

Ključne riječi: *Balkanska nefropatija; Apoptoza; Nefron; Stereologija*