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Analysis of apoptosis of kidney tissue by the tunel method and histomorphological changes in rabbit kidney model due to unilateral supravesical obstruction

Tavşan böbrek modelinde tek taraflı supravezikal tıkanıklığa bağlı böbrek dokusunda oluşan apoptozisin tunel yöntemiyle analizi ve histomorfolojik değişiklikler

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Abstract

Aim: There is a need for effective, cheap, and fast method to detect apoptosis. In some studies, we see that newer, more difficult, expensive, or less effective methods are used. We wanted to show that the TUNEL method has serious advantages and can still be used alone. In this study, it was aimed to investigate whether there is a significant difference in the number of apoptotic cells in partial obstruction (PO) and complete obstruction (CO) by using terminal deoxytransferase-mediated bio-dUTP nick and labeling (TUNEL) method in renal tissue. We also evaluated histopathological changes after renal obstruction.

Methods: In this study, 29 rabbits were used. Supravesical obstruction was created in 24 rabbits. Five rabbits were used as the control group. Twelve kidneys were examined after creating unilateral partial obstruction and 12, after the creation of unilateral complete obstruction. Histomorphological changes in kidney tissues in routine Haemetoxylene-Eosine (HE) preparations and apoptosis in preparations obtained by TUNEL method were examined.

Results: Apoptotic cells were observed especially in the tubules by the TUNEL method. The average number of apoptotic cells in CO and PO groups were 190.66 and 40.58, respectively. In the CO group, the number of apoptotic cells was significantly higher than that in the PO group ($P<0.001$). Interstitial fibrosis, chronic inflammatory infiltration, tubular destruction (vacuolar changes, cystic and atrophic tubules) were observed in both groups. These changes were more limited, and mild, in the PO group, and severe and widespread in the CO group.

Conclusion: TUNEL method is one of the highly effective methods in detecting apoptosis. It was observed that apoptosis and pathological changes developing in the kidney tissue after complete obstruction were more severe and widespread.

Keywords: Renal obstruction, Apoptosis, TUNEL method

Öz

Amaç: Literatürde, bazı çalışmalarda, daha yeni, ama pratikte değerlendirme güçlükleri olan, pahalı veya etkinliği az yöntemlerin kullanıldığını görüyoruz. Terminal deoxytransferase-mediated bio-dUTP nick and labeling (TUNEL) yönteminin ciddi avantajları olduğunu ve halen tek başına kullanılabileceğini göstermek istedik. Bu nedenle, deneysel olarak tavşan üreterinde kısmi ve tam obstrüksüyon oluşturulmasından sonra böbrek dokusunda gelişen apopitozisin şiddetinin, TUNEL yöntemi kullanılarak belirlenmesi, gruplar arasında anlamlı bir fark olup olmadığı ve yanısıra oluşan histopatolojik değişiklikleri değerlendirme amaçlandı.

Yöntemler: Mesane üstü seviyesinde üreter geçişinin kısmi ve tam olarak engellenmesi yoluyla-12 tam obstrüksiyon, 12 kısmi obstrüksiyon grubu-, 5 taneside kontrol grubu olmak üzere- toplam 29 adet tavşan böbreği kullanıldı. Böbrek dokusunda gelişen histomorfolojik değişiklikler Hematoksilen-Eozin (HE) preperatlarda, apoptozis ise TUNEL yöntemiyle hazırlanan preperatlarda mikroskopik olarak incelendi. Obstrüksiyon sonrası oluşan apopitozisin tam ve kısmi obstrüksiyon grupları arasında istatistiksel olarak anlamlı farklılığı olup olmadığı araştırıldı.

Bulgular: Apopitotik hücreler, TUNEL yöntemiyle net olarak gösterildi. Apopitozisin, özellikle tübül epitelinde yoğunlaştığı görüldü. Tam obstrüksiyon grubunda, ortalama apopitotik hücre sayısı 190,66 ve kısmi obstriksüyon grubunda ise 40,58 idi. Tam obstrüksiyon grubunda apopitotik hücre sayısı kısmi obstrüksiyon grubuna göre anlamlı derecede yüksekti ($P<0,001$). 2 grupta da interstisyel fibrozis, kronik iltihabi infiltrasyon, tübüler yıkım (vakuoler değişiklikler, kistik ve atrofik tübüller) gözlendi. Bu değişiklikler kısmi tıkanma grubunda daha sınırlı, hafif, tam tıkanma grubunda şiddetli ve yaygın idi.

Sonuç: TUNEL yönteminin apopitozisi belirlemede etkinliği yüksek yöntemlerden biri olduğu gösterildi. Tam tıkanma sonrası böbrek dokusunda gelişen apopitozisin ve patolojik değişikliklerin çok daha şiddetli ve yaygın olduğu görüldü.

Anahtar kelimeler: Renal obstrüksiyon, Apopitozis, TUNEL yöntemi

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Introduction

Apoptosis was first described in 1972 by Austrian pathologist John Kerr [1]. It is an active system associated with many genes and has a definition similar to leaf dump in Greek due to combination of the words apo (= separate) and ptosis (= falling). Understanding the mechanisms involved in apoptosis in mammalian cells was triggered by the investigation of programmed cell death during the development of nematode Caenorhabditis elegans [2]. Apoptosis is a cell death pathway in which cells activate enzymes that breakdown their nuclear DNA and nuclear / cytoplasmic proteins. The resulting cellular debris breaks away from the main structure and apoptosis, which means "falling of leaves," occurs. It may develop during physiological events such as organogenesis and tissue growth or may be induced by pathological stimuli. Therefore, the mechanisms leading to apoptosis have attracted attention and been extensively studied. For this purpose, studies investigated what role it plays in the mechanisms of human diseases through experimental studies in many animal models [3].

Renal obstruction due to the deterioration of tissue nutrition is one of the important problems. The role of apoptosis in this process has been investigated in many studies. In addition, in various experimental studies, the differences in apoptotic cell density after partial and / or complete obstruction of the kidney and the mechanisms of apoptosis have been investigated or reviewed [4-11]. The mechanisms and causes of apoptosis development are important, so it is necessary to determine the apoptosis developing in the tissue clearly and healthily. There are many methods with high sensitivity and can be applied to determine apoptosis. The TUNEL method is one of the most commonly used methods in determining apoptosis. It is a system that detects DNA fractures by enzymatic reaction. The reaction with the TUNEL method is highly descriptive and only the nuclei of the apoptosis are stained. The method is based on the specific binding of Tdt to the 3-OH ends of the DNA following synthesis of the polyoxynucleotide polymer and enables the detection of DNA fractures within the cell. With this method, the presence of apoptotic cells can easily be detected in sections obtained from paraffin blocks where the sampled tissues are embedded. It is an important advantage that preparations can be evaluated with light microscopy [12-20].

In this study, we aimed to investigate whether there is a significant difference in the number of apoptotic cells in partial and complete renal obstruction by the TUNEL method. We also evaluated histopathological changes after renal obstruction. In addition, the TUNEL method is used in studies investigating whether apoptosis occurs and whether it is involved in the mechanisms responsible for the development of different lesions. In addition, the TUNEL technique is used in studies where active substance use and treatment effectiveness are evaluated. Therefore, it was aimed to evaluate the effectiveness and usability of TUNEL method in terms of contribution to the literature.

Materials and methods

A total of 29 animals (New Zealand adult rabbits with an average weight of 1500-2000 grams) were included in the study. They were fed standard diet [18% protein, 3% fat, 20% fiber foods and 10% carbohydrates, 0.009 mg / kg vitamin complex (A-B-C-E)]. Animals were divided into three groups: Unilateral complete obstruction group (12 rabbits), unilateral partial obstruction group (12 rabbits), control group (5 rabbits). Sodium pentobarbital (0.04mg / kg) was injected into the peritoneum. After cleaning and dressing under sterile conditions, the skin was shaved from the midline abdomen. The layers were passed through a 13-15 cm long, midline abdominal incision. The bleeding was quickly controlled with 4/0 vicryl. Full obstruction left ureteropelvic obstruction was performed by suturing the ureter with 5/0 silk 4-5 cm distal to the renal pelvis. Partial obstruction was created by passing through the ureter tunnel and approaching the edges close to each other by creating a 15 mm tunnel to the psoas muscle. After bleeding control, the layers were closed in the anatomical plane. Following intraperitoneal administration of the same dose of anesthetic agent to the control group, the longitudinal abdominal incision was closed with 4/0 vicryl. One month later they were sacrified with a high-dose anesthetic agent. Left and right kidneys were excised according to technique by providing sterile conditions. The left and right kidneys of 12 rabbits in two groups and the left kidney of 5 animals in the control group were fixed with a 10% buffered formaldehyde solution. After the samples of kidney tissue including cortex and medulla were obtained, tissue follow-up was performed. Following this procedure, 5 micron thick sections were taken from the tissues embedded in paraffin blocks and two preparations were used from each case. Histopathological changes in HE preparations after obstruction were examined. The other one was stained by TUNEL method to show apoptotic cells using Oncor's Apoptag Peroxidase kit-cathologist no: S7100- USA (Equilibration Buffer, Reaction Buffer, TdT Enzyme, Stop/Wash Buffer, Anti-Digoxigenin-Peroxidase). In TUNEL method preparations, especially after apoptosis of renal tubular epithelial cells, dense chromatin localized to the nucleus was stained as light-dark brown. Apoptotic cell number / 1000 cells were determined at high magnification (x400) by light microscope. Pathological evaluation was done blindly by two different pathologists. Apoptotic cell count was recorded in 12 cases in both groups.

Statistical analysis

SPSS 15.0 package program was used for statistical analysis of the data. Categorical measurements were compared in numbers and percentages, continuous measurements were averaged, deviations were compared between groups, the distribution was checked, and Mann Whitney U test was used in binary variables. In all tests, $p<0.05$ was considered statistically significant.

Results

Histopathological findings in complete obstruction group

Ischemic changes in obstructed kidneys were evaluated in HE sections. Hydronephrotic changes were observed macroscopically. Most tissues had interstitial fibrosis and mononuclear inflammatory cell infiltration. Calcification and steatosis were observed in one case. Dilatation and vacuolar degeneration were detected in tubules. Cystic atrophy was

observed in some tubules. Thickening of the vessel walls and erythrocyte accumulation in some vessel lumens were observed. In addition, inflammatory infiltration, which concentrates around the vessels and disrupts the endothelium in some places, was noticed (Figure 1, 2).

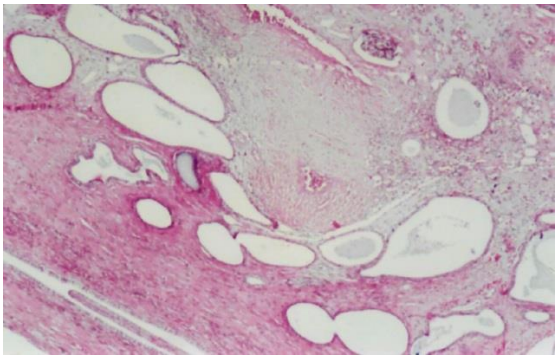

Figure 1: Cystic / atrophic tubules and severe fibrosis in renal tissue after complete obstruction, HE x40

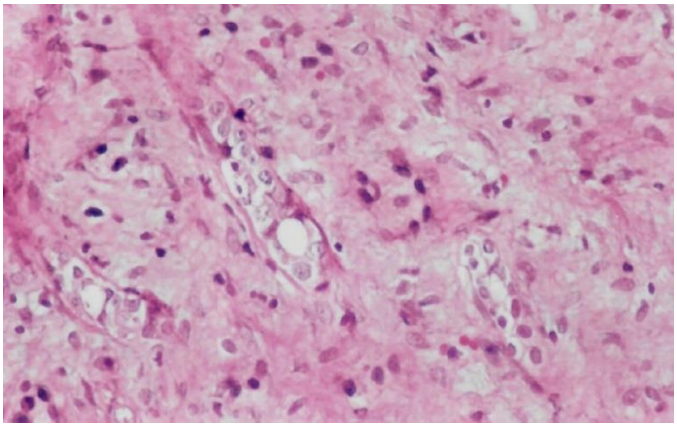

Figure 2: Atrophic renal tissue with a small number of tubules with severe fibrosis after complete obstruction and vacuolar degeneration in the epithelium, HE x100

Histopathological findings in partial obstruction group

In some cases, interstitial fibrosis and mononuclear inflammatory cell infiltration were observed. In some cases, inflammatory cell infiltration was intense in the perivascular region. Some vessels had erythrocyte accumulation in the lumen. In one case, there was bleeding and necrosis in the parenchyma. Some of the tubules were distorted and mild nuclear degeneration and vacuolization were observed in epithelial cells (Figure 3).

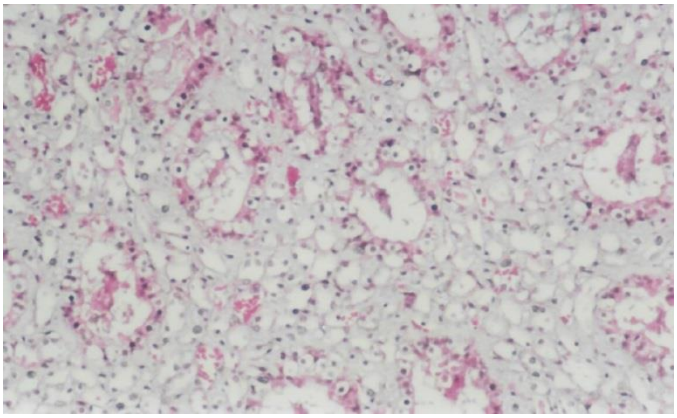

Figure 3: Congestion in the renal tissue after partial obstruction, dense vacuolar degeneration in the tubular epithelium, HE x40

Fibrosis and tubular involvement were more severe in completely obstructed kidneys.

It was emphasized that there was no significant pathological finding in the control kidney sections.

Findings in TUNEL method

The lowest number of apoptotic cells in the CO group was 3/1000, the highest was 441/1000 (Table 1) (Figure 4). These values were 2/1000 and 170/1000 (Table 2), respectively, in the PO group (Figure 5, 6). The average number of apoptotic cells in the CO and PO groups were 190.66 (160.62) and 40.58 (58.63), respectively. The number of apoptotic cells in the kidneys in the CO group was significantly higher than those in the PO group (P<0.001). Apoptotic cells were rarely seen in the control group.

Table 1: Number of apoptotic cells determined in rabbit kidneys in CO group

Complete obstruction (CO) group	Apoptotic cell count (n/1000)
1	3
2	3
3	3
4	51
5	66
6	210
7	219
8	242
9	335
10	350
11	365
12	441

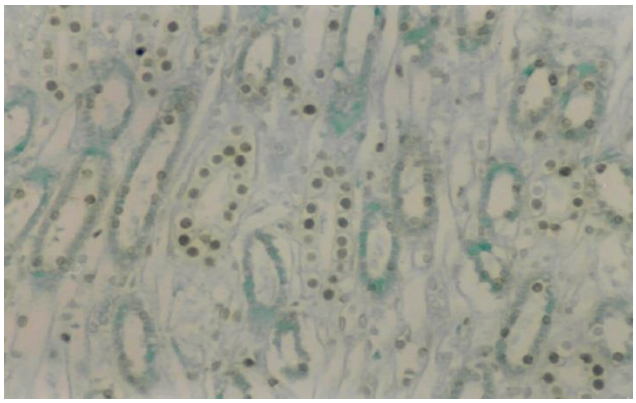

Figure 4: Light-dark brown nuclear staining, showing the presence of frequent apoptosis in tubules after complete obstruction, TUNEL, x100

Table 2: Number of apoptotic cells determined in rabbit kidneys in the PO group

Partial obstruction (PO) group	Apoptotic cell count (n/1000)
1	2
2	2
3	4
4	5
5	6
6	7
7	7
8	18
9	47
10	78
11	141
12	170

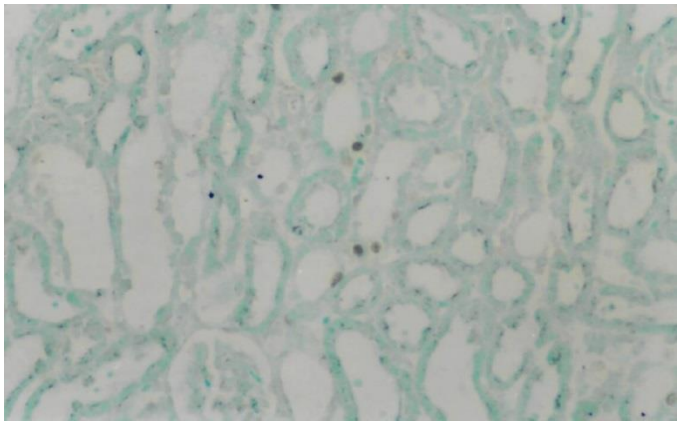

Figure 5: Presence of rare apoptosis in tubules after partial obstruction, light-dark brown nuclear staining, TUNEL, x100

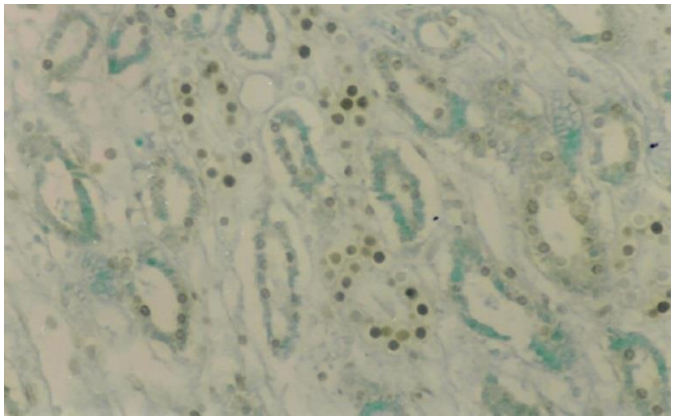

Figure 6: Presence of frequent apoptosis in tubules after partial obstruction, light-dark brown nuclear staining, TUNEL, x100

Typically, apoptotic cells in kidney tissue were observed in the proximal and distal tubules in the deep part of the cortex and in the medulla layer, and in the lining epithelial cells in the collection ducts. Apoptosis is rarely seen in the vascular walls and interstitium. In TUNEL method preparations, apoptotic cells were identified as light-dark brown stained areas localized to the nucleus, which were clearly separated from the cytoplasm, especially in tubules, on a green background.

Discussion

Obstructive uropathy due to unilateral ureteral obstruction (UUO) has characteristic functional and structural changes. UUO is characterized by tubular cell damage, interstitial inflammation and fibrosis due to hydrostatic pressure caused by obstruction. Therefore, molecular mechanisms of apoptosis have been investigated as a model of events that occur during irreversible acute kidney injury and human chronic kidney disease [4]. In the first few days after ureteral obstruction, a decrease in GFR, a decrease in kidney blood flow, interstitial edema and leukocyte infiltration are observed. Hydronephrosis and tissue loss develop over time, pronounced tubular atrophy, interstitial fibrosis and interstitial inflammation ocur [4-8]. In experimental models evaluating tubulointerstitial pathologies developing after obstruction, tubular epithelial cell damage and interstitial inflammation are observed in the acute phase. If this tubular injury cannot be regenerated, necrosis or apoptosis and subsequent scar tissue may occur. The most important morphological changes in this process are atrophy, focal necrosis, epithelial regeneration, apoptosis, inflammation, interstitial fibrosis, and thrombosis development [21,22]. Apoptosis is stimulated in the tissue exposed to oxygen metabolites after acute renal ischemia. In conclusion, tubules and blood vessels are the main targets for injury due to renal ischemia and are usually observed in the most severe external medulla [5,7,22]. Although the most serious involvement in our cases was in the medulla layer, the cortical layer was affected and the concentration of apoptotic cells in the tubules was remarkable. In our study, apoptosis, interstitial fibrosis, chronic inflammation, and tubular atrophy were observed in all kidneys, especially in the CO group, due to obstruction. In some cases, focal necrosis and thrombosis were seen. The most severe destruction occurs in the outer medulla layer and especially in the tubules due to the characteristics of normal circulation.

Tubular epithelial cells are responsible for high transport activity. This function is supported by mitochondrial function, oxygen depletion affects the mitochondrial function severely. Intensive studies of the mechanism of tubular destruction have highlighted 3 mechanisms of epithelial cell loss: Necrosis, loss of cell integrity, and apoptosis. Ischemia affecting cell functioning is the basis of the formation of these 3 mechanisms. The common finding of ischemia is tubular basement membrane rupture. Nephron involvement probably comes later. Afterwards, local inflammatory response occurs in the environment. Because of these data, studies on inhibition of tubular cell apoptosis have begun and it has been shown that kidney damage can be stopped [23].

In investigations for the control of apoptosis, stimulation and prevention mechanisms were investigated. It is known that three different precursor types of signaling pathways are involved in the induction of apoptosis. Mitochondria / cytochrome-C mediated apoptosis is induced by death activators binding to cell surface receptors, and endoplasmic reticulum mediated apoptosis. In general, molecules such as calcium, ceramide, bcl-2 family, p53, caspases, proteins such as cytochrome c and mitochondria play a role in the regulation of apoptosis. Naturally, the balance mechanisms between cell proliferation and apoptosis are regulated and managed at the molecular level through mediators. Some of these have been understood in experimental models [7,9-11,24]. Apoptotic pathways active in the glomerular and tubular epithelium include survival factor deprivation, death receptor activation, mitochondrial damage, endoplasmic reticulum stress, lysosomal destabilization, and caspase cascade activation. These pathways are interrelated but show stimulus-specific differences [25]. Various cytokines and growth factors, particularly TNF-alpha [26], osteopontin [27], TGF-1, angiotensin, nuclear factor-κB (NF-κB), are involved in the development of occlusion-induced renal fibrosis and apoptotic cell death [28]. In addition, Omi / HtrA2 has been shown to be associated with apoptotic signaling pathways in tubular epithelial cells affected by unilateral ureteral obstruction [29]. Over time, the mechanisms of apopitosis-related proteins were understood and new ones were discovered. The main proteins associated with apoptosis in the kidney tissue formed in UUO include Angiotensin 2, Caspases, Fas-L, ICAM-I, IL-6, TGF-beta, VCAM-I, MCP-I, TNF-alpha, and the proapoptotic-proinflammatory mechanism. Nitric oxide, Heat shock protein-70 and COX-2 have antiapoptotic effects. Also, mediators (TGF-beta, SMA, Vimentin, PDGF, Integrin (β1), PAI-1, TIMP-1, CTGF) are known to cause fibrosis and exacerbate the process leading to loss of function in the obstructed kidney [30]. Apoptotic cell death is usually a response to the cell's microenvironment, and this response requires the activation of molecules that bring death to the cell or the inactivation of prosurvival molecules that prolong the life of the cell. Both are currently potential therapeutic targets [25]. Many active ingredients are tried for achieving these goals. Among them, calcium channel blockers that have previously been shown to be effective [31], fluuorofenidone which inhibits collagen-1 overexpression leading to renal fibrosis, indirectly antiapoptotic effect [32], angiotensin-2 receptor antagonists fimasartan [33], erythropoietin receptor, bcl -2, erythropoietin, which has antiapoptotic effects by suppressing bcl-x1 mRNA release [34], rhein, which is thought to suppress the bax and bcl2 proteins

associated with apoptosis [35], colchicine, which is suggested to be antiapoptotic by suppressing caspase-3 and fibronectin [36], bcl-2, molecules such as troxerutin [37], which is effective on many proteins such as bax, TNF-alpha, and Ulinastatin [38], which is used in the treatment of acute pancreatitis and whose antiapoptotic effect has been tested in the cell damage that occurs in the brain, has been shown to reduce the pathological degeneration in the tissue by affecting the mechanisms that cause apoptosis in the UUO kidney.

Apoptosis is an established mechanism not only to control cell number and tissue size, but also to remove infected, damaged or stressed cells from the organism. Therefore, the ability to detect and manage apoptosis is essential in the control and treatment of diseases [13]. Determination of apoptosis was originally based on morphological criteria. Later, DNA breaks were identified by finding that which caspases were activated. Currently used methods are based on morphological, immunohistochemical, immunological, biochemical, and molecular biology [13,17-20]. These include light microscopy, special / fluorescent dyes, immunohistochemistry, electron microscopy, PCR, TUNEL, DNA agarose gel electrophoresis, Flow cytometry, In situ3 - end labeling method (ISEL), Western blotting and caspase colorimetric assay, Nuclease assay [20]. The most common methods are Spectroscopy, Electron Microscopy, Electrophoresis, immunohistochemistry (staining with Annexin V, Caspase 3, p53, M30), TUNEL and Flow cytometry [13].

Morphological evaluation in light microscopy, although easy and cheap, is subjective and is not preferred because of low reproducibility. Evaluation in electron microscopy is a highly accurate method, but it is time consuming, expensive and a small area of tissue can be examined. Determination of apoptosis by electrophoresis is advantageous in terms of being easy, precise, and quantitative. However, living, apoptotic or necrotic cell differentiation may not occur due to the damage it can cause to the cell membrane. Flow cytometry evaluates live and fixed cells individually, easily, quickly and accurately. It is sensitive to the amount of apoptosis. It is time consuming due to multi-step and enzymatic precursor processes. Immunohistochemistry can be preferred as it is easy and inexpensive. The biggest problem is that the methods used require separating, washing and transferring the cells. These procedures can damage cell membranes and alter the cell population distribution of viable, apoptotic and / or necrotic cells [13,17-20].

TUNEL is based on the direct marking of the 3 hydroxyl ends of DNA breaks and therefore the measurement can be defined directly at the molecular level. DNA breaks occur very early in apoptosis, so the method also detects apoptotic cells that are not yet recognized based on changes in morphology. Therefore, it gives the opportunity to determine the first reactions of apoptosis. In addition to DNA breaks, DNA content can also be measured. However, the disadvantage is that the sensitivity and specificity of this technique depend on the concentration of fixative and terminal transferase enzyme used [17]. Today, there are many experimental studies using the UUO model, especially for treatment. While evaluating apoptosis in these, the combination with TUNEL and newer techniques (especially western blotting, flow cytometry and immunohistochemical examinations) are preferred. In these studies, the apoptosis indices obtained in the TUNEL method complement each other with the results of other new techniques and provide information on the efficacy of the molecules investigated for treatment [35,39,40]. In addition, TUNEL method is used alone and effectively in many experimental study models other than the UUO model. In our study, the clarity of the data supports this [41,42].

The strengths of the study are the large number of subjects, the use of control subjects, the use of the surgical technique by the experienced urologist, no technical problems in the application of the TUNEL method, and the blind evaluation of the results by two different pathologists.

Limitations

The lack of comparative analysis of the results we obtained using the TUNEL method with another current and effective method limits the value of the study.

Although we think that the TUNEL method can be used alone in experimental studies investigating the therapeutic efficacy of active ingredients in this field, it may be preferable to use combination with new techniques that confirm the robustness of the results.

In our study, apoptotic cells were clearly demonstrated by the TUNEL method. Technical artifact was minimal and excluded from evaluation. As expected, the number of apoptotic cells in CO was much higher than that in PO. There was a statistically significant difference in the number of apoptotic cells between the two groups. In complete obstruction, the severity of apoptosis and associated pathological changes, fibrosis and atrophy were more intense than partial obstruction.

Conclusions

Apoptosis is a phenomenon that has been handled in many aspects, is still up-to-date, and is used mostly to direct therapeutic research. It continues to be examined especially on the basis of the UUO model in terms of its formation mechanisms and methods of determination. With the passage of time, although there are many new apoptosis assessment methods, the TUNEL method continues to be used alone or in combination with other current methods, due to its applicability and reproducibility, as well as not being too time consuming and expensive.

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