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Evaluation of renal damage biomarkers in an experimental Pyelonephritis model ınduced by uropathogenic *Escherichia coli*

Evaluación de los biomarcadores de daño renal en un modelo experimental de Pielonefritis inducida por *Escherichia coli* uropatógena

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ABSTRACT

This study aims to determine the levels of biomarkers in rats on different days of the disease by creating a pyelonephritis model using Uropathogenic *Escherichia coli* (UPEC). Forty rats were used in the study; 10 were designated as the control group and the remaining 30 rats were intrarenally administered UPEC to create a pyelonephritis model. Blood and urine samples were collected on days 1, 4 and 7 of the experiment. Histopathologically, it was determined that pyelonephritis occurred in all experimental groups. In serum samples, significant changes were observed in the groups' clusterin, L-FABP and clusterin/Cr levels. In urine samples: while no significant changes were detected in Cr, clusterin, NGAL/Cr and clusterin/Cr levels, significant alterations were identified in NGAL, L-FABP, KIM-1, cystatin C, KIM-1/Cr, cystatin C/Cr and L-FABP/Cr levels. In the scope of the study, changes in the identified biomarkers in the serum and urine samples of rats with induced pyelonephritis were particularly evident. In evaluations conducted on different days of the disease, it was observed that urine NGAL, L-FABP, KIM-1 and cystatin C levels increased up to the 4th day compared to the control group. These findings suggest that urine biomarkers, in particular, may play a significant role in diagnosing pyelonephritis.

Key words: Kidney; pyelonephritis; uropathogenic *Escherichia coli*; biomarker; rat

RESUMEN

El objetivo de este estudio es determinar los niveles de biomarcadores en diferentes días de la enfermedad en ratas, creando un modelo de pielonefritis mediante el uso de *Escherichia coli* Uropatógena (UPEC). Se utilizaron cuarenta ratas en el estudio; 10 fueron designadas como grupo de control y a las 30 ratas restantes se les administró UPEC de forma intrarrenal para inducir el modelo de pielonefritis. Se recolectaron muestras de sangre y orina los días 1, 4 y 7 del experimento. Desde el punto de vista histopatológico, se determinó que la pielonefritis ocurrió en todos los grupos experimentales. En las muestras de suero, se observaron cambios significativos en los niveles de clusterina, L-FABP y en la relación clusterina/Cr entre los grupos. En las muestras de orina, aunque no se detectaron cambios significativos en creatinina, clusterina, NGAL/Cr y la relación clusterina/Cr, se identificaron alteraciones significativas en los niveles de NGAL, L-FABP, KIM-1, cistatina C, KIM-1/Cr, cistatina C/Cr y L-FABP/Cr. Dentro del alcance del estudio, los cambios en los biomarcadores identificados en las muestras de suero y orina de ratas con pielonefritis inducida fueron particularmente evidentes en las muestras de orina. En las evaluaciones realizadas en diferentes días de la enfermedad, se observó que los niveles de NGAL, L-FABP, KIM-1 y cistatina C en orina aumentaron hasta el cuarto día en comparación con el grupo de control. Estos hallazgos sugieren que, en particular, los biomarcadores en orina pueden desempeñar un papel significativo en el diagnóstico de la pielonefritis.

Palabras clave: Riñón; pielonefritis; *Escherichia coli* uropatógena; biomarcador; rata

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INTRODUCTION

Urinary tract infections (UTIs) can occur in any part of the urinary system. The most common types include asymptomatic bacteriuria, acute uncomplicated cystitis and pyelonephritis [1]. Uropathogenic *Escherichia coli* (UPEC) is the most common cause of these infections [2 , 3 , 4]. Pyelonephritis, which develops when bacteria travel from the bladder to the kidneys and can lead to serious complications, poses diagnostic challenges due to the lack of specific symptoms [5 , 6]. Therefore, the importance of biomarkers in the diagnostic process is increasingly recognized.

Cystatin C is a protein found in various body fluids such as urine, plasma and saliva. Due to its stable structure, it has been proposed as an alternative to creatinine (Cr) for measuring glomerular filtration rate. It is widely used in acute and chronic kidney diseases [7 , 8 , 9].

Clusterin (as a molecular chaperone) binds to misfolded proteins and directs them for lysosomal degradation, regulating the protein-folding process [10 , 11]. Several studies have shown that clusterin has a higher diagnostic power in detecting kidney injury, especially proximal tubular damage than standard markers such as blood urea nitrogen and serum Cr [12 , 13].

Neutrophil gelatinase-associated lipocalin (NGAL), a protein capable of detecting acute kidney injury (AKI) more quickly and accurately than serum Cr, also plays a role in combating bacterial infections [14]. Numerous studies have demonstrated that NGAL is more sensitive than serum Cr in identifying AKI and suggested its potential use as a beneficial biomarker in clinical practice [15 , 16].

Liver-type fatty acid-binding protein (L-FABP) is an effective indicator for detecting oxidative stress and tubulointerstitial damage in the kidneys by facilitating the excretion of lipid peroxidation products [17 , 18]. In clinical studies, the urinary level of this biomarker has been used to predict the progression of kidney disease [19 , 20].

Kidney injury molecule 1 (KIM-1), a protein highly expressed in kidney tissue, has emerged as an important biomarker for the early diagnosis of AKI and cardiovascular diseases and for monitoring the treatment process [21]. However, it has also been reported that KIM-1 levels fluctuate significantly in cats with AKI compared to healthy cats [22].

This study aims to determine the levels of KIM-1, NGAL, L-FABP, clusterin and cystatin C, as well as changes in Cr, urea, total protein and albumin levels; it does so by tracking these markers on different days. Specifically, it focuses on assessing specific biomarkers associated with acute and chronic kidney injury in an experimentally induced pyelonephritis model.

MATERIALS AND METHODS

Forty non-pregnant adult female Sprague-Dawley rats (*Rattus norvegicus*), 12 weeks old and weighing 250 ± 50 (OHAUS-Navigator) grams, were used in the study. The rats were kept under conventional conditions at 22 ± 1°C with a 12–hour light/dark cycle. They were divided into four groups, each with 10 animals.

The Local Ethics Committee approved the study for Animal Experiments of Aydın Adnan Menderes University (Protocol no: 2022/031).

Preparation of bacteria

The UPEC was stored at –20°C (Bosch KDV4200NE, Germany) until the day of the experiment. The night before the study, the bacteria were revived in an incubator (Memmert, Germany) set to 37°C. The revived bacteria were centrifuged (Nuve, Nf 800R, Turkey) at 7440 *g* for 3 min, washed three times and resuspended in sterile physiological saline solution (0.9% NaCl) [23].

Experimental study

This study was conducted at Aydın Adnan Menderes University, Faculty of Veterinary Medicine, Experimental Animals Production and Research Center. The rats were divided into four groups (1 control group and 3 experimental groups), with 10 animals in each group. Ten rats were assigned to the control group, while the remaining rats were anesthetized intraperitoneally with xylazine (Interchemie) -ketamine (Doğa İlaç). The abdominal area of the anesthetized rats was shaved, disinfected with alcohol (70%) and povidone-iodine (10%) and prepared for surgery. A midline incision was made in the upper abdominal region to expose the right kidney.

To induce experimental pyelonephritis, a freshly prepared 100 µl bacterial suspension (UPEC-ATCC 25922, 1×10^9 CFU) was injected into the right kidney cortex and directed towards the medulla using a 1 mL syringe. Before returning the kidneys to the abdominal cavity, they were rinsed with sterile physiological saline. The abdomen was closed and the rats were returned to their cages [1 , 24 , 25].

In Group 1, blood samples were collected intracardially, one d after the injection; in Group 2, four d after; and Group 3, seven d after. Blood samples were also collected from the control group at the end of the study. Rats were anesthetized intraperitoneally with a combination of ketamine (100 mg/kg) and xylazine (10 mg/kg) [26] and blood samples were collected according to established protocols [27].

Serum and urine clusterin, NGAL, L-FABP, KIM-1 and cystatin C levels were measured using the ELISA, while total protein, urea, Cr and albumin levels were measured spectrophotometrically (Shimadzu UV-1601, China).

Histopathological examination of kidneys

At the end of the experiment, the right and left kidneys of the animals in the control and experimental groups were collected. The kidneys were bisected longitudinally using a scalpel and fixed in 10% buffered formalin. The fixed kidneys were promptly sent to a contracted laboratory.

Tissues were processed in an automatic tissue processor (Leica TP1020, USA), following a routine procedure that included dehydration in graded alcohols, clearing in xylene (98,5%) and embedding in paraffin. Thin sections (5-6 µm) of paraffin-embedded tissues were prepared using a microtome (Leica RM2125 RTS, USA) and then stained with hematoxylin and eosin [28]. Using a light microscope (Carl Zeiss Axiolab 5, China), the prepared slides were evaluated and microphotographs (Olympus DP27, Japan) were taken for documentation [28].

Statistical analyses

The obtained data was analyzed using SPSS (Statistical Package for Social Sciences) for Windows version 21. The normality of the data distribution was tested with the Shapiro–Wilk method. Differences between groups with normal distribution were analyzed using one-way analysis of variance (ANOVA) and the significance of these differences was tested using post hoc Tukey and Tamhane tests. For datasets not meeting the normality assumption, group differences were examined via the Kruskal–Wallis test. The Bonferroni-adjusted Mann-Whitney U test was performed to determine the source of the differences. Statistical findings with a p-value below 0.05 were deemed significant and all results are presented as mean ± standard error.

RESULTS AND DISCUSSIONS

The results of the serum samples are presented in TABLE I; the urine samples are presented in TABLE II. Serum Cr, urea, albumin and total protein levels significantly increased in the study groups than the control group.

In the urine, while Cr and urea levels decreased, albumin and total protein levels increased. Statistical analysis indicated that the changes in urea, albumin and total protein levels were significant, whereas the change in Cr level was not.

TABLE I. Mean serum biochemical values in rats with renal damage biomarkers in a UPEC-induced experimental Pyelonephritis model

Parameters	n	Control	Day 1	Day 4	Day 7	P
Creatinine (mg/dl)	10	0,43±0,029^{ab}	0,44±0,0,035^{ab}	0,33±0,017^{a}	0,50±0,029^{b}	0,011*
Urea (mg/dl)	10	0,48±0,05^{a}	1,19±0,15^{b}	1,00±0,06^{b}	1,12±0,07^{b}	0,0001***
Albumin (g/dl)	10	2,22±0,055^{a}	3,49±0,143^{ab}	3,91±0,040^{b}	3,77±0,067^{b}	0,0001***
Total Protein (g/)dl	10	4,43±0,22^{a}	5,82±0,33^{b}	7,06±0,20^{c}	7,48±0,42^{c}	0,000***
Cystatin C (ng/mL)	10	10,87±0,57^{a}	10,08±0,48^{a}	10,57±0,44^{a}	11,59±0,62^{a}	0,269 NS
Clusterin (ng/mL)	10	28,74±0,32^{a}	29,95±0,63^{ab}	32,76±2,17^{b}	33,72±1,61^{b}	0,003**
NGAL (ng/mL)	10	10,11±0,55^{a}	10,65±0,37^{a}	10,53±0,74^{a}	10,35±0,44^{a}	0,906 NS
L-FABP (ng/mL)	10	31,62±2,21^{a}	22,39±1,55^{b}	24,06±1,75^{b}	24,25±1,04^{b}	0,003**
KIM-1 (ng/mL)	10	1,411±0,049^{a}	1,182±0,073^{a}	1,235±0,048^{a}	1,282±0,088^{a}	0,119 NS
Cystatin C/Cr (ng/mg Cr)	10	2366,03±302,02^{a}	3694,46±932,06^{a}	2867,51±455,21^{a}	2854,51 ±305,76^{a}	0,624 NS
Clusterin/Cr (ng/mg Cr)	10	5724,94±745,64^{a}	10692,96±2431,47^{ab}	10079,12±615,64^{b}	8640,31±706,34^{ab}	0,007**
NGAL/Cr (ng/mg Cr)	10	2206,30± 294,90^{a}	3874,71±730,22^{a}	3017,57±561,99^{a}	2549,01±357,10^{a}	0,197 NS
L-FABP/Cr (ng/mg Cr)	10	7358,59±825,07^{a}	7616,21±1629,38^{a}	7793,52±749,75^{a}	6008,32±289,99^{a}	0,511 NS
KIM-1/Cr (ng/mg Cr)	10	382,96±94,46^{a}	421,19±103,49^{a}	341,44± 56,03^{a}	306,33±34,02^{a}	0,853 NS

Neutrophil gelatinase-associated lipocalin: NGAL, Liver-type fatty acid-binding protein: L-FABP, Kidney injury molecule 1: KIM-1, Creatinine: Cr. All data are presented as means ± standard error. Different letters ^{abc} in the same row indicate significance (P < 0.05). *** indicates P < 0.001, ** indicates P < 0.01, * indicates P < 0.05 and NS indicates no significant difference between groups.

TABLE II. Mean urine biochemical values in rats with renal damage biomarkers in a UPEC-induced experimental Pyelonephritis model

Parameteres	n	Control	Day 1	Day 4	Day 7	p
Creatinine (mg/dl)	10	72,72±8,01^{a}	77,40±13,39^{a}	50,90±9,74^{a}	60,25±3,69^{a}	0,202 NS
Urea (mg/dl)	10	8,74±0,76^{a}	5,79±0,53^{b}	6,29±0,47^{b}	7,42±0,70^{ab}	0,013*
Albumin (g/dl)	10	0,103±0,012^{a}	0,152±0,030^{ab}	0,175±0,023^{ab}	0,177±0,010^{b}	0,007**
Total Protein (g/dl)	10	0,29±0,03^{a}	0,26±0,05^{a}	0,50±0,07^{ab}	1,33±0,40^{b}	0,0001***
Cystatin C (ng/mL)	10	7,57±0,68^{a}	12,74±0,75^{bc}	13,59±0,52^{b}	10,57±0,42^{c}	0,0001***
Clusterin (ng/mL)	10	99,46±18,79^{a}	80,13±17,47^{a}	97,36±7,87^{a}	72,44±4,91^{a}	0,233 NS
NGAL (ng/mL)	10	8,76±0,52^{ac}	11,51±0,78^{b}	10,78±0,44^{ab}	7,33±0,54^{c}	0,0001***
L-FABP (ng/mL)	10	20,74±2,23^{a}	31,86±3,52^{ab}	29,26±2,10^{b}	21,94±1,48^{a}	0,005**
KIM-1 (ng/mL)	10	0,805±0,048^{a}	1,224±0,077^{b}	1,221±0,049^{b}	0,990±0,053^{a}	0,0001***
Cystatin C/Cr (ng/mg Cr)	10	9,69± 2,05^{a}	25,86± 5,38^{ab}	26,96± 5,62^{b}	20,73± 2,13^{ab}	0,013*
Clusterin/Cr (ng/mg Cr)	10	112,66±22,88^{a}	147,03±29,70^{a}	159,41±44,71^{a}	131,85±5,79^{a}	0,385 NS
NGAL/Cr (ng/mg Cr)	10	10,89± 2,17^{a}	23,00±4,58^{a}	25,59±5,87^{a}	15,10±1,60^{a}	0,077 NS
L-FABP/Cr (ng/mg Cr)	10	25,23±5,89^{a}	62,95±13,56^{b}	58,31±15,38^{b}	43,63±5,51^{b}	0,041*
KIM-1/Cr (ng/mg Cr)	10	0,94± 0,09^{a}	2,52±0,58^{b}	2,33±0,58^{ab}	1,93±0,19^{b}	0,017*

Neutrophil gelatinase-associated lipocalin: NGAL, Liver-type fatty acid-binding protein: L-FABP, Kidney injury molecule 1: KIM-1, Creatinine: Cr. All data are presented as means ± standard error. Different letters abc in the same row indicate significance (P < 0.05). *** indicates P < 0.001, ** indicates P < 0.01, * indicates P < 0.05 and NS indicates no significant difference between groups.

Many studies evaluating humans and animals with kidney diseases classify cystatin C as a helpful biomarker [29 , 30 , 31]. Studies investigating pyelonephritis have reported different results regarding changes in serum cystatin C levels. Some studies on this subject [32 , 33 , 34] have reported that serum cystatin C levels in patients with acute pyelonephritis were significantly higher than those without. In contrast, Islekel *et al*. [35] stated no significant change in serum and urinary cystatin C levels in pyelonephritis patients. In this study, while no significant difference was detected among the groups in terms of serum cystatin C and cystatin C/Cr levels, a statistically significant increase was observed in urinary cystatin C and cystatin C/Cr levels. These findings align with studies indicating that urinary cystatin C levels strongly correlate with kidney function in kidney diseases. The results indicate that urinary cystatin C levels may be a valuable biomarker for diagnosing pyelonephritis.

In the literature search, no study determined serum and urine levels of clusterin, except for a histopathological study examining the relationship between clusterin and pyelonephritis cases. In that histopathological study by Sansanwal *et al*. [36], weak immunohistochemical staining was observed in the kidneys of patients with pyelonephritis. Weak staining indicates that clusterin is expressed at low levels in pyelonephritis. In our study, serum clusterin and clusterin/Cr ratios were significantly elevated in the pyelonephritis groups compared to the control group. The fact that the increase in serum clusterin/Cr ratio was more pronounced than the increase in serum clusterin levels suggests that evaluating these two parameters together may be more useful in diagnosis.

In a canine endotoxemia model designed to simulate kidney injury caused by *Escherichia coli* (*E. coli*)-derived lipopolysaccharides, it was reported that AKI occurred and urinary clusterin and urinary clusterin/Cr levels significantly increased before any rise in serum Cr levels [37]. In this study, *E. coli* was used to induce pyelonephritis and some similarities were observed between the current model and the canine model. However, unlike the model applied in dogs, no significant increase was detected in urinary clusterin and clusterin/Cr levels. Additionally, the absence of a significant increase in urinary clusterin and clusterin/Cr levels is inconsistent with previous studies on AKI [12 , 13].

Neutrophil gelatinase-associated lipocalin is a widely used biomarker for diagnosing urinary system diseases such as chronic kidney diseases and urinary tract obstruction.Studies evaluating NGAL as an indicator of kidney damage highlight the sensitivity of urinary NGAL and NGAL/Cr ratios in detecting AKI [37 , 38 , 39]. This study showed a notable increase in serum NGAL/Cr, urinary NGAL and urinary NGAL/Cr levels, especially on the first day. While the increases in serum NGAL/Cr and urinary NGAL/Cr levels were not statistically significant, the increase in urinary NGAL levels was substantial. These results suggest that NGAL measurement may be a diagnostic indicator for acute pyelonephritis rather than a prognostic biomarker.

Within the limited number of studies on the levels of L-FABP in pyelonephritis, contradictory findings exist. In one study, it was emphasized that L-FABP levels do not help identify pyelonephritis [40]. In contrast, other studies reported that urinary L-FABP and urinary L-FABP/Cr levels increase in children with pyelonephritis [41] and significant rise in urinary L-FABP in urinary tract infections [42 , 43]. Among the parameters we evaluated, serum L-FABP levels significantly decreased, whereas the L-FABP/Cr ratio changes were not statistically significant. The increase in urinary L-FABP and L-FABP/Cr ratio in the pyelonephritis groups is consistent with the literature, more studies are needed to examine the relationship between pyelonephritis and L-FABP.

Studies in humans and animals have reported varying results regarding serum and urinary KIM-1 levels. Krzemien *et al*. [44] and Urbschat *et al*. [45] suggested that urinary KIM-1 is ineffective in detecting pyelonephritis. In contrast, Lee *et al*. [46] and Rius-Gordillo *et al*. [47] reported a significant increase in urinary KIM-1/Cr levels during the acute phase of the disease. In this experimental pyelonephritis model, serum KIM-1 levels decreased over time; however, this change was not statistically significant. Skowron *et al*. [48] developed a pyelonephritis model in rats using different doses of UPEC and investigated its dose-dependent effects. Similarly, in the pyelonephritis group induced with 1×10^9 bacteria, urinary KIM-1 levels were reported to have increased significantly within one week. In this study, consistent with the findings of Skowron *et al*., a significant increase in urinary KIM-1 levels was observed in the pyelonephritis group compared to the control group. Studies have shown that urinary KIM-1 is more specific than serum or plasma KIM-1 and may serve as a noninvasive biomarker for early diagnosis [49]. Similarly, the findings of this study support that urinary KIM-1 levels could be a valuable indicator for the diagnosis of pyelonephritis.

The histopathological findings are presented in detail in FIG. 1. In the histopathological evaluation, pyelonephritis was found in the kidneys of all animals in the experimental group, while no pathological findings were detected in the control group (FIGS. 1. A, B).

In rats belonging to the first- and fourth-d groups, dense neutrophilic leukocyte infiltration, focal microabscess formation, mild lymphocytic infiltration in the renal interstitium and thickening of the glomerular basement membrane were observed. In tubular epithelial cells, severe vacuolization, marked tubular lumen dilatation, hyaline cast formation in some dilated tubules, tubular atrophy and intense neutrophilic leukocyte infiltration in the collecting ducts were noted. Signs of urine stasis were detected in the cortical tubules. Squamous hyperplasia and leukocyte infiltration were observed in the transitional epithelium of the renal pelvis (FIGS. 1. C, D, E).

In rats belonging to the seventh-d group, intense lymphoplasmacytic cells, less dense neutrophilic leukocyte infiltration and widespread fibrosis were observed in the interstitium. Extensive necrotic areas and severe glomerulosclerosis were noted in some rats. Glomeruli and tubules were utterly absent in parenchymal areas where fibrosis was prevalent. In highly atrophied tubules and glomeruli, thickening of the basement membrane was observed. Squamous hyperplasia and inflammation dominated by lymphocytes were observed in the transitional epithelium of the renal pelvis (FIGS. 1. F, G, H).

In this study, the changes in potential biomarkers of kidney diseases and traditional diagnostic parameters in pyelonephritis patients were investigated, yielding the following findings. In serum samples, clusterin and clusterin/Cr levels significantly increased, while L-FABP markedly decreased. In urine, significant increases were detected in the levels of NGAL, L-FABP, KIM-1, cystatin C, KIM-1/Cr, cystatin C/Cr, and L-FABP/Cr. Among the routine biochemical parameters evaluated, Cr, urea, albumin, and total protein levels showed significant increases in serum, whereas in urine, all parameters except Cr exhibited significant changes.

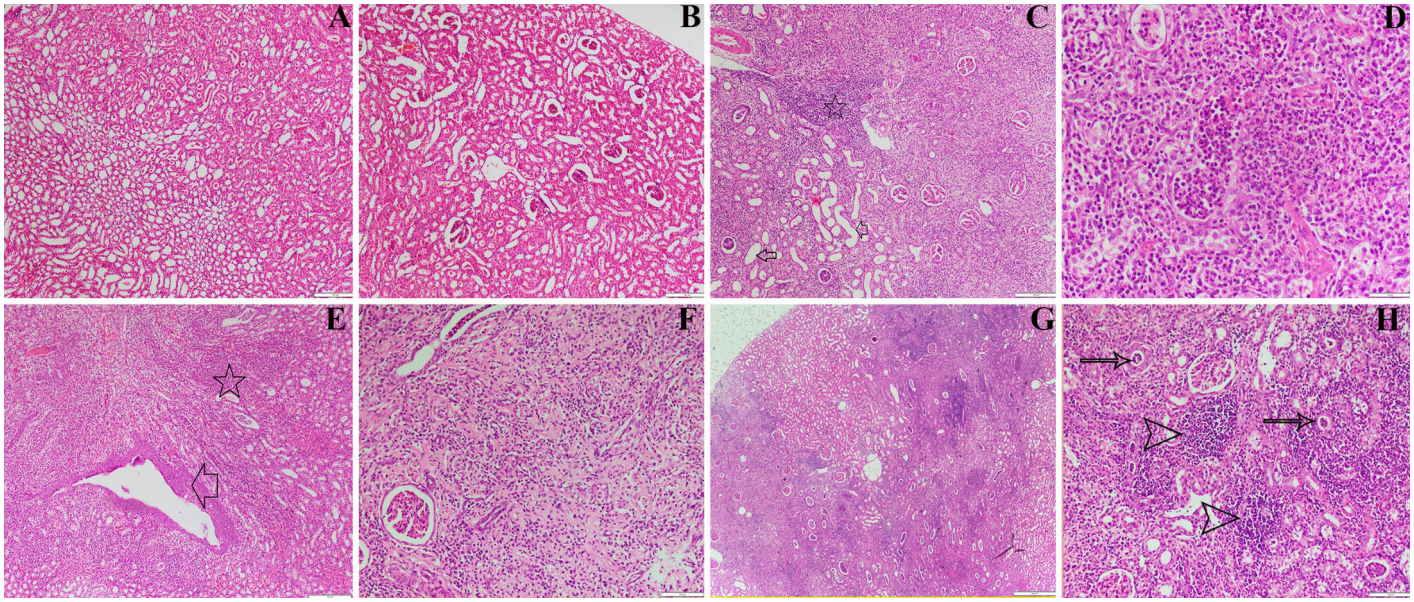

FIGURE 1. Histopathological findings in an experimental Pyelonephritis model. A) Control group medulla (Bar: 200 µm). B) Control group cortex (Bar: 200 µm). C) 1 d group. Widespread neutrophil infiltration (star) and tubular dilatation (arrow) (Bar: 200 µm) D) 4 d group. Neutrophil infiltration in tubules (Bar: 50 µm) E) 4 d group. Squamous hyperplasia (arrow) in pelvic epithelium with leukocyte infiltration (star) (Bar: 200 µm). F) 7 d Group. Widespread fibrosis, glomeruli and tubules are lost (Bar: 500 µm) G) 7 d group. Widespread fibrosis, glomeruli and tubules are lost (Bar: 200 µm) H) 7 d group. Lymphocyte infiltration (arrowheads) in the interstitium of the cortex and tubules (arrows) and atrophy (Bar: 100 µm). Hematoxylin and eosin stain.

CONCLUSION AND RECOMMENDATIONS

In conclusion, the obtained findings suggest that urinary biomarkers may potentially be superior in the diagnosis of pyelonephritis. Additionally, clusterin, investigated for the first time in the serum and urine samples of pyelonephritis patients, showed significant increases in serum clusterin and clusterin/Cr levels, indicating its diagnostic value. However, the lack of significant changes in urinary clusterin levels highlights the need for further research into its role. These findings support the potential of urinary biomarkers for clinical use in pyelonephritis diagnosis, while more comprehensive studies are required to clarify their diagnostic and prognostic value in the future.

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Conflict of Interest Statement

The authors declare no conflict of interest.

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