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LABORATORY DIAGNOSTICS OF RENAL FUNCTION IN AN EXPERIMENTAL MODEL OF ASCENDING PYELONEPHRITIS WITH HIGH-VIRULENT ESCHERICHIA COLI

Abstract: *Laboratory diagnostics of renal function in an experimental model of ascending pyelonephritis with high-virulent Escherichia coli.*

Introduction: Urinary tract infections (UTI) are caused in 95% of cases by bacteria — E. coli. UTIs usually are limited to the lower urinary tract, but it may also evolve into pyelonephritis and acute kidney injury.

Objectives: The aim of this study was the laboratory evaluation of renal function in an experimental model of ascending pyelonephritis caused by intravesical infusion of E. coli.

Material & Methods: In female Wistar rats UTI was induced by intravesical administration of E. coli suspension in a dose 10^5 c.f.u./ml (*Group 1*), and 10^7 c.f.u./ml (*Group 2*). On the 0,7th, 14th and 21st day of the experiment the animals underwent the procedures of collecting blood and urine samples.

Results: The results shown that in *group 2* on the 7th and 14th day of the study the creatinine clearance decreased by 36%, and on 21th by 34%. The increase in serum uric acid concentration (μmol/l) in *group 2* was observed on the 7th (229.75 ± 79.05) and 21st day (98.5 ± 11.33) with respect to day 0 (77.12 ± 11.63). In *group 2* on the 7th day of the experiment there was observed the increased levels of potassium (mmol/l) in serum (13.5 ± 1.48) with respect to day 0 (7.74 ± 0.88). In *group 2* in the 7th (1.06 ± 0.18) and 14th day (1.32 ± 0.26) there was noted the decreased excretion of potassium in the urine (mmol/24h) with respect to day 0 (3.75 ± 1.9). The decrease in serum sodium levels (mmol/l) in *group 2* was recorded on 14th day (121.5 ± 8.7) with respect to day 0 (131.62 ± 4.07). Increased factional sodium excretion — FENa (%) was observed in *group 2* on 14th day (0.25 ± 0.06) with respect to day 0 (0.12 ± 0.06).

Conclusions: Our main finding is that — independently of the amount bacteria present in urinary bladder — in this inflammatory model there occurs inevitably acute kidney injury, however higher bacteria amount depicts a very clear profile of laboratory parameters that point at the kidney impaired function.

Key words: pyelonephritis, Escherichia coli, kidney function, rat

1. INTRODUCTION

Urinary tract infection (UTI) is a bacterial infection that affects parts of the urinary tract, which are otherwise sterile under physiological conditions [1]. Due to differences in the anatomy, UTIs affect mostly women. Pyelonephritis (PN) is a form of UTI that has reached the pelvis of the kidney. It is a form of nephritis that is also referred as pyelitis. The increased incidence of UTIs occurs especially during sexual activity in women using vaginal inserts. Also, in pre- and postmenopausal period, as a consequence of hormonal imbalance, there is observed a resultant decreased urethral sphincter tonus that is leading to frequently recurrent episodes of UTIs [2].

The source of bacteria that are responsible for 95% of non — hospital UTIs, both in women and men, is the digestive tract. For 90% of UTIs cases the etiological factor is Escherichia coli (group: Enterobacteriaceae). E. coli is responsible for about 50% cases also of UTIs in hospitalized patients. The other etiological agents isolated in urine culture are, as follow: Klebsiella, Proteus, Enterobacter, Enterococcus faecalis and Staphylococcus aureus, fungi (*e.g.* Candida albicans), Chlamydia trachomatis, Neisseria gonorrhoeae, and viruses (*e.g.* Herpes simplex) [3].

UTIs usually are limited to the lower urinary tract. Statistics show that only 1 in 28 cases (< 5%) undergoing uncomplicated cystitis will develop secondary pyelonephritis. The bacteria, colonizing the urinary bladder, are moving to the upper compartments *via* insufficient antireflux system of intramural section of ureters. Kidney infection undergoes from the core, spreading to the adjacent parts of the cortex. The presence of the pathogens in the renal parenchyma causes the development of inflammation and immune activation processes that are changing the hemodynamic status of the kidney and lead-to its functional impairment [4]. In case of UTIs, there is a risk of acute interstitial nephritis, which is one of the causes of acute kidney injury (AKI), and in some cases leads to chronic kidney disease [5].

Assessment of the impaired renal function is based mainly on the laboratory analysis of urine and blood samples. The specific studies allow to determine the degree of organ damage and are useful to monitor the efficacy of precautionary treatment. One of the most frequently performed studies that are being used for evaluation of renal function is the assessment of glomerular filtration rate (GFR) by measuring creatinine clearance (CrCl). Due to the fact that creatinine is excreted only with urine and its serum concentration correlates inversely with GFR value, creatinine is considered as a marker of kidney function [6]. In addition to the diagnostic importance of creatinine clearance, in assessing the degree of renal damage there has also being used uric acid. Similarly to creatinine, uric acid is also excreted mainly by renal filtration (70%). The increase of the serum uric acid concentration, resulting from a decrease in

glomerular filtration, leads through the process of crystallization to tubular obstruction and local inflammation. These properties of uric acid are pointing at this substance as for an important marker of acute kidney injury detection and assessment [7]. Often, the increase in plasma — both the creatinine and uric acid — is associated with electrolyte imbalance. Kidneys are the most important organs in sodium/potassium homeostasis. Spreading of the disease process leads to disturbed homeostasis and is observed as changes in electrolytes levels. Dyselectrolitemia in the course of kidney disease can lead to several complications, including arrhythmia, paralytic ileus, mental deterioration, etc. Hyperkalaemia is the most common cause of death in AKI and therefore parameters such as serum potassium and sodium concentrations must be strictly monitored in this condition [8].

2. AIM

The aim of this study was the laboratory evaluation — with standard renal function tests — of an experimental dynamic model of ascending PN caused by intravesical injection of high-virulent Escherichia coli strain.

3. MATERIAL AND METHODS

3.1. Animals

The tests were performed on ten (10 weeks old) female Wistar rats that weighed approximately 200g (205.1 ± 10.9 g). Animals came from the animal facility of Faculty of Pharmacy, Jagiellonian University in Krakow. Animals were kept in a carefully maintained hygienic conditions in an air conditioned room with constant temperature 21–25°C, with preserved twelve-hour day-night cycle and *ad libitum* access to water and food. The experiment was approved by Local Ethical Committee of Jagiellonian University, Cracow, Poland (resolution No 133/2012).

3.2. Research groups and research protocol

According to the project's assumptions, the animals were divided in two groups:

Group 1. Animals with UTI induced by intravesical infusion of 0.5 ml suspension of E. coli strain with high virulence in a dose of 10^5 c.f.u./ml.

Group 2. Animals with UTI induced by intravesical infusion of 0.5 ml suspension of E. coli strain with high virulence in a dose of 10^7 c.f.u./ml.

The animals from both groups underwent the following procedures:

Procedure 1. **Induction of ascending UTI.** On the zero-day of experiment, the animals in general anesthesia (urethane anesthesia, Urethane, Sigma--Aldrich, administered i.p., in a dosage of 200 mg/kg body weight) underwent a procedure of placing a catheter into the bladder (sterile polyethylene catheter with a diameter of 1 mm and length 10 cm). Through the catheter there was given in two doses 0.5 ml suspension of bacteria E. coli with high virulence, respectively: g*roup 1* was infected with a dose 10^5 c.f.u./ml, *group 2* was infected with a dose 10^7 c.f.u./ml. The procedure of evoking the ascending UTI was carried out, after modifications, according Lee *et al.* [9].

Procedure 2. **Blood sampling.** On zero, 7th, 14th and 21st day of the experiment, the animals in general urethane anesthesia underwent a procedure of blood sampling. Blood was collected from the tail vein — each sample 0.5 ml of blood. The samples were then prepared for the biochemical analysis.

Procedure 3. **Urine sampling.** In order to collect urine for biochemical studies, animals on zero, 7th, 14th and 21st day of the experiment were placed in previously sterilized (steam sterilizer, type ASHE/A, 121°C, 15 min.) metabolic cages (metabolic cage for rats 150–300 g, Tecniplast, Italy). The animals' residence time lasted 24 h.

Procedure 4. ***Post mortem*** **examination.** On 21st day of the experiment the animals were sacrificed by an overdose of anesthetic agent — sodium pentobarbital (Morbital, Biowet Pulawy, i.p. administration, in a dosage of 100 mg/kg body weight). Then there was performed a dissection, the kidneys and bladder were removed and placed in formalin solution (4% formalin in phosphate buffer solution (PBS pH 7.4) for further histological evaluation. The dissection was performed under sterile conditions in a laminar air flow, using previously sterilized surgical tools (steam sterilizer, type ASHE/A, 121°C, 15 min.), as well as aseptic tools (sterile gloves, swabs, gauze).

3.3. Characteristics of E. coli

The strain that was used in our study was isolated from a patient with pyelonephritis. It was characterized by having genes for several virulent factors: *fimH, papC, sfaD/E* — which encode adhesins type 1 and fimbriaes type P,S. Strain was having also *iroN* gene, responsible for encoding salmocheline — ferric ions receptor, and *cnf-1* gene, responsible for encoding TNF1 [10].

3.4. Laboratory analysis

During the study there were analyzed: creatinine clearance, serum uric acid, sodium and potassium in urine and serum, and fractional excretion of sodium.

All parameters analyzed in the urine were calculated on the basis of the amount of urine collection. The concentrations of ions in both — the serum and urine — were conducted on a flame photometer, model Corning 450. Other parameters were measured with using the biochemical analyzer Olympus AU 600.

3.5. Histological evaluation

Organs prepared during dissection were rinsed in saline, then fixed for 24 hours in 4% formalin in phosphate buffer solution (PBS pH 7.4). The preparates were made for evaluation under a light microscope. After deparaffination procedure, they were cut with using of microtome for slides with a thickness of 3–5 microns. Then the preparates were stained with hematoxylin — eosin (HE) staining procedure. Preparates were evaluated using a magnification of 100, 200 and 400×.

3.6. Statistics

Statistical analysis of the results was performed using Statistica 5.5 (StatSoft). The results for each group are shown as mean with standard deviation. For comparison the differences between the analyzed groups, there was used ANOVA test. Tukey's HSD test was used to identify the detailed differences between analyzed groups. The differences between groups were considered statistically significant for a probability level of $p < 0.05$. As the control for each group there were assumed the results obtained from zero-day, where the average value of the results of all 10 animals used in the experiment (there was no differences observed between the 0 day in both groups).

4. RESULTS

The study was performed on 10 animals, weighing 205.1 ± 10.9 g that were divided into two study groups. Between research groups, there was no statistical significant differences in initial body mass ($p = 0.39$).

4.1. Histological evaluation

In the histological preparates obtained from bladders there was observed the hyperinflation of the micro-vascular bed in *group 1* and *2*, and inflammatory infiltration specific just for *group 2*. In the preparates obtained from the kidneys, both in *group 1* and *2,* there was observed mononuclear cells chronic inflammatory infiltration.

4.2. Laboratory analysis

4.2.1. Creatinine clearance

Table 1

Creatinine clearance values during the experiment;
p values > 0.05 indicate that creatinine clearance did not differ significantly in both groups

Parameter	Day	Group 1	Group 2	p value
Creatinine clearance (ml/min.)	0.	1.52 ± 0.53	1.52 ± 0.53	–
	7.	1.86 ± 0.51	**0.98 ± 0.18**	**0.055**
	14.	1.14 ± 0.34	0.97 ± 0.13	NS
	21.	1.03 ± 0.25	1.01 ± 0.13	NS

The p value that equals 0.055 indicates a tendency to gain statistical significance that could imply the need of further experiments on the basis of a larger group of animals. In comparison to the day "zero" of the experiment, the creatinine clearance in *group 2* decreased in a manner of 36% for the 7th and 14th day and of 34% for the 21st day.

4.2.2. The serum concentration of uric acid

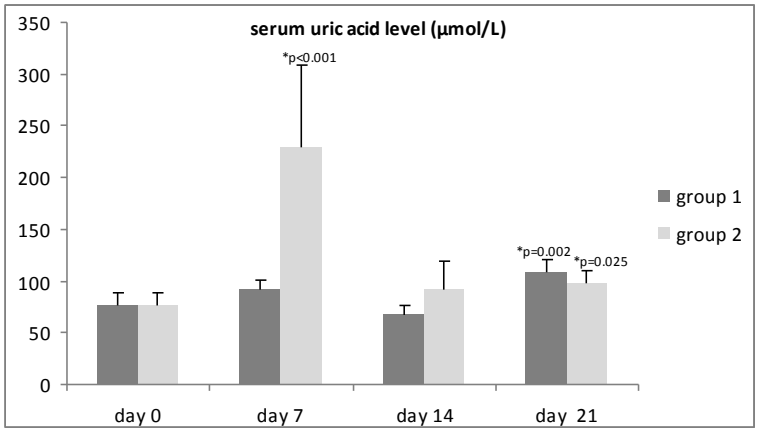

Fig. 1. The serum concentration of uric acid during the experiment;
p values < 0.05 for the 7th and 21st day indicate that the experimental groups differ statistically significant
** vs. day "zero" of the experiment*

4.2.3. The serum potassium level

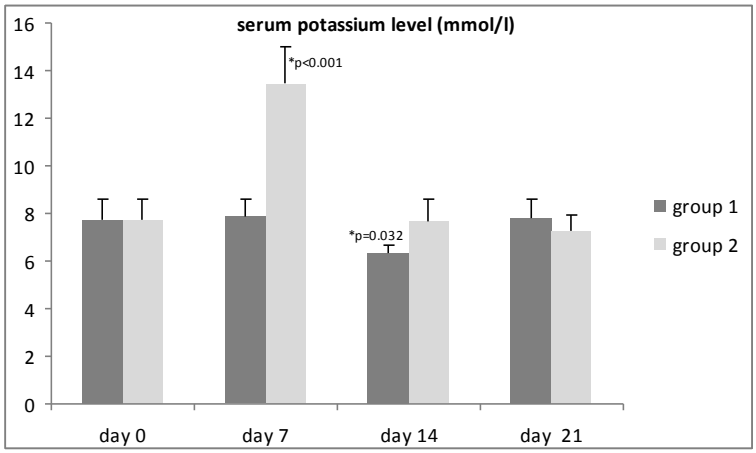

Fig. 2. The serum potassium level during the experiment;
p values < 0.05 for the 7th and 14th day indicate that the experimental groups differ statistically significant
** vs. day "zero" of the experiment*

4.2.4. The potassium excretion in the urine

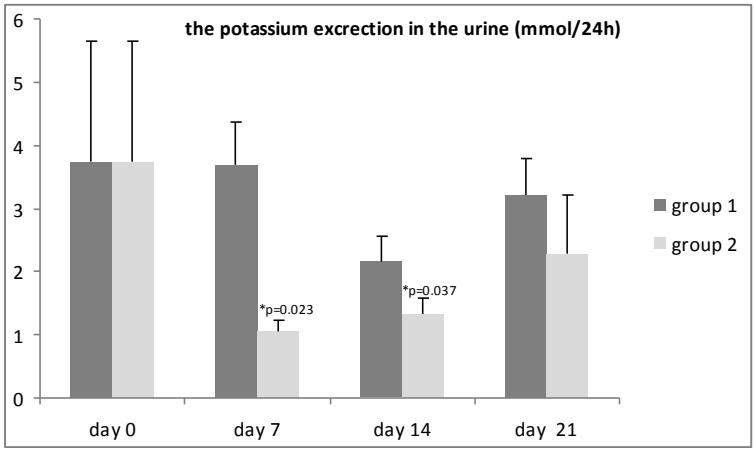

Fig. 3. The potassium excretion in the urine during the experiment;
p values < 0.05 for the 7th and 14th day indicate that the experimental groups differ statistically significant
** vs. day "zero" of the experiment*

4.2.5. The serum sodium level

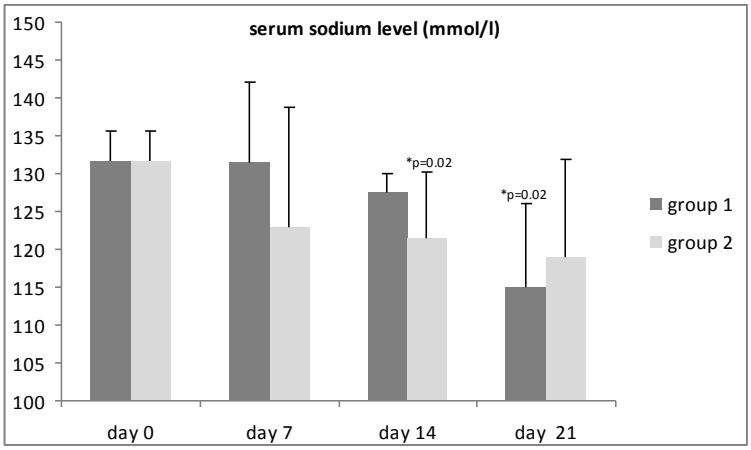

Fig. 4. The serum sodium level during the experiment;
p values < 0.05 for 14th and 21st day indicate that the experimental groups differ statistically significant
** vs. day "zero" of the experiment*

4.2.6. The sodium excretion in the urine

Table 2

The sodium excretion in the urine during the experiment;
p values > 0.05 indicate that the experimental groups did not differ statistically significant

Parameter	Day	Group 1	Group 2	p value
The sodium excretion in the urine (mmol/24h)	0.	0.35 ± 0.17	0.35 ± 0.17	–
	7.	0.29 ± 0.15	0.31 ± 0.07	NS
	14.	0.31 ± 0.27	0.44 ± 0.17	NS
	21.	0.21 ± 0.09	0.26 ± 0.08	NS

4.2.7. The fractional sodium excretion (FENa)

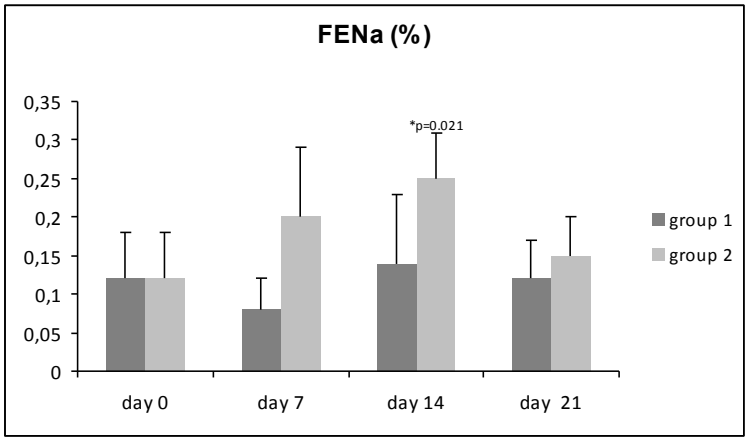

Fig 5. The fractional excretion of sodium during the experiment;
p values < 0.05 for the 14th day indicates that the experimental groups differ statistically significant
** vs. day "zero" of the experiment*

5. DISCUSSION

The results of this experiment show the dynamics of the inflammation induced process of the kidney injury caused by two different doses of E. coli with high virulence strain. Selection of the etiological agent used in the experiment was closely related to the epidemiology of urinary tract infections, which in most patients is caused by a E. coli that predominate in the colon.

Histological evaluation. Histological studies have confirmed that within 21 days after intravesical bacteria injection the kidneys of animals of both groups developed features of chronic inflammation with mononuclear cells inflammatory infiltration. Severe injury was confined to the inner medulla and comprises features of tubular damage and scarring. These changes suggest that there was a passage of bacteria from urinary bladder to the upper sections of the urinary tract, which in turn resulted in the activation of the inflammatory process within the kidney and impair their function. The vast majority of changes observed during the experiments occurred in *group 2*, infected with high bacteria concentration.

CrCl. On the 7th day of experiment in *group 2*, there was a reduction in creatinine clearance in a manner of 36%. Additionaly, GFR value at 64–66% was maintained up to 21 days after infection. The equalization of GFR in *group 1* to

a similar level as in *group 2* occurred within 21 days. These data indicate that the dynamics of pathological changes in the urinary tract were dose-dependent and inevitable. Increase of serum creatinine levels caused by a decrease in GFR on 7th day after infection was described by Sener *at al.* in experimental model of pyelonephritis caused by injection of bacteria in the kidney tissue. E. coli injected at dose of 10^{10} c.f.u./ml increased five times creatinine level [11]. Malekinejad *at al.* in similar studies, after kidney tissue injection of the E. coli at dose of 10^6 and 10^7 c.f.u./ml observed increase of creatinine level by 30% and 40% in 3rd and 14th day after injection [12]. Thus tissue injection was more effective then bladder instillation. Heyman *at al.* in a different model of kidney injury — developing of AKI after administration of radiocontrast, in uninephrectomized, salt-depleted rats, injected with indomethacin. After 24 h plasma creatinine level was doubled and the creatinine clearance dropped from 0.7 to 0.2 ml/min. [13]. In our model, E. coli instillation at a dose of 10^7 c.f.u./ml, resulted in increased creatinine level only in a manner of 20% and GFR decreased of 36% in 7th day. These results suggest that decrease in kidney function /damage/ is strictly dose — and administration route dependent.

Uric acid. Uric acid metabolic pathway ends up almost entirely in the proximal tubule. The accuracy of all of the markers based on the proximal tubules function, requires theirs intact function. In particular, uric acid excretion will increase (independently of volume status) when proximal reabsorption is reduced [14]. And so confirms our study, where we observed an increase in *group 2* in the early in the course of the experiment. The decrease in GFR in a manner of 36% on the 7th day causes over 100% increase in serum uric acid level in *group 2*. The elevated level of serum uric acid was maintained until the last day of the study in *group 2*, whereas in *group 1*, there is no such phenomena and a significant increase in serum uric acid concentration appears as a single peak on 21st day after instillation. Fact that serum uric acid is a marker of kidney damage was observed in surgical patients, when blood flow and GFR decreased, uric acid level increased [15]. Increased uric acid level of about 1 mg/dl increases risk of kidney damage of about 74% [16]. Its reduced excretion, as a consequence of declining GFR, causes the crystallization within the renal tubule and enhancement of the inflammatory processes [17]. Proinflammatory effect of uric acid occurred by activation of MCP-1 (*Monocyte Chemoattractant Protein-1*), CRP (*C-reactive protein*) and p38 MAPK (*Mitogen--activated Protein Kinase*). Besides proinflammatory effects of the uric acid, there was shown also its pro-oxidative feature. The elevated level of serum uric acid inhibits the NOS1, and — in a consequence — reduces NO and stimulation of the RAA system. Therefore, such cascade, may induce the damage of blood vessels in the kidneys and impairment of their function [18]. In our experiment, changes in the serum uric acid levels were correlated with the decrease of GFR

and both of the changes are dose-dependent when it's about the concentration of E. coli injections. Highest increase of serum uric acid level occurred on 7th day of infection in *group 2*.

Electrolytes. Observed in our study decline in GFR value in this particular model of kidneys' injury induced also changes in water balance and electrolytes abnormalities [19]. Tubular ions transport is impaired by mediators of inflammation. The most important pro — inflammatory cytokines and mediators are in that case IL-1 and PGE2 that reduce activity of Na-K-ATPase and induce changes in kidney hemodynamics [20]. Hiperkalaemia and hyponatraemia — observed in our *group 2* — confirmed these observations that the cause of the electrolytes abnormality is renal dysfunction [21]. Electrolytes imbalance occurred not only in pyelitis but also in all cases of AKI [22]. Significant changes in serum potassium levels appear in our experiment early on the 7th day of the experiment. The increase in serum potassium excretion correlates with the daily urine production, as well as with the decrease of GFR value. Hiperkalaemia that was resulting from the GFR value decrease, is a characteristic symptom of chronic kidney injury. Hsieh *at al.* found that decrease of the eGFR value of about 10ml/min increases serum potassium level of about 0,117 mmol/l [23]. Hyponatraemia has occurred after two weeks of the experiment and was correlating with a fractionated sodium excretion that indicates increased renal damage [24].

FENa. The FENa is considered the most accurate clinical diagnostic test to differentiate between prerenal and renal disease like acute tubular necrosis (ATN) that are considered as two most common causes of AKI [25]. The FENa and the urine sodium concentration are usually measured to determine if a patient's effective fluid volume isn't depleted [26]. In our experiments lowered level of serum sodium concentration occurred only in *group 2* after two weeks of infection. The relatively higher FENa in *group 2* of our experiments can be observed as a result of one or both of the following factors: inappropriate sodium wasting due to tubular inflammatory damage and an inappropriate response to volume expansion of fluids. The former is most likely to be important early in the disease when nephrons that are still filtering have impaired tubular function. Volume expansion may be more important in late form of pyelonephritis with established tubular damage and normal tissue perfusion in the healthy regions. At the end of inflammatory process most of the damaged nephrons are excluded and nonfunctioning. Urine output results from a number of well--preserved nephrons. In this period, the maintenance of body sodium balance requires a higher FENa [27].

The newest data are pointing at a lack of information about relationship between dose of bacteria E. coli and biomarkers of kidneys' function. Our main finding is that — independently of the amount of bacteria present in urinary bladder — in this inflammatory model there occurs inevitable kidney injury.

Moreover, higher bacteria concentrations cause the more pronounced increase in the laboratory markers of renal function.

Acute renal failure may develop after the noxious stimulus response within a few hours to several days [28]. In our experiments, in both groups, this time ranges from 1 to 14 days. The standard parameters analyzed in the experiment, like: the decrease of creatinine clearance, the increase of serum uric acid level, the increase of serum potassium and the decrease of potassium urine level were observed within 7 days after infection. After two weeks, sodium concentration imbalance occurs with the decreased serum sodium levels and increased excretion of fractional sodium. In order to answer the question — how fast is the dynamics of the inflammatory process and at what dose the etiological agent is needed to see advanced AKI — it is necessary to repeat the experiments with using new markers of AKI, as well as to monitor the renal function parameters that would be based on shorter time intervals.

CONFLICT OF INTERESTS STATEMENT

None declared.

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