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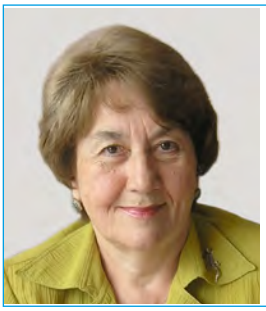
KIDNEY UREAPLASMOSIS IN TERMS OF EVIDENCE MEDICINE (EXPERIMENTAL STUDY)



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Our aim was to create an experimental model of pyelonephritis in animals using Ureaplasma parvum and to examine the histology of renal pathological process as well as its biochemical and immunological characteristics. Modeling of the rabbits' medical condition was based on V. Pratt method in our own adaptation. Observation was conducted for over 90 days with the studying of biochemical and immunological indices changes.

Using U.parvum when creating pyelonephritis experimental model in rabbits caused the development of the kidney lymphoid cell infiltration, microcirculation disorders, stroma swelling, initial vessels and interstitium sclerosis as well as the development of the degenerative changes in the epithelium of the kidney tubular system and urothelium bladder.

Animals infected with ureaplasma had hematogenous dissemination of the pathogen in different organs and generalized ureaplasmic infection, which caused systemic immune response in the form of specific antibodies titres increasing to ureaplasma. We proved the development of systemic inflammation in the greater part of parenchymal organs (uterus, liver, lungs, heart, thyroid gland) with the most florid onset in the infected kidneys and lungs. Biochemical indices time course reconstituents a pattern of changes in the kidneys, bladder, liver, lungs, uterus and other organs.

U.parvum should be considered as a predictor of acute inflammation of a kidney and urinary tract caused by classical bacteria on the one hand and a development of pathological changes that can be considered as a primary chronic inflammatory process, which differs from the bacterial on the other.

Key words: *experimental ureaplasmic pyelonephritis, ureaplasma, generalized ureaplasmic infection.*

Метою роботи було створити експериментальну модель пієлонефриту на тваринах за допомогою Ureaplasma parvum, вивчити гістологічну картину патологічного процесу нирок, біохімічні й імунологічні показники. Моделювання захворювання у кролів базувалось на методиці V. Prat у власній модифікації. Спостереження проводилось протягом 90 діб із вивченням динаміки біохімічних та імунологічних показників.

Застосування U.parvum при створенні експериментальної моделі пієлонефриту у кролів призвело до розвитку у нирках лімфоїдно-клітинної інфільтрації, порушень мікроциркуляції, набряку строми, початкового склерозу інтерстиція та судин, розвитку дистрофічних змін в епітелії канальцевої системи нирки та уротелії сечового міхура.

У тварин, інфікованих уреаплазмами, відбувалася гематогенна дисемінація збудника у різні органи й розвивалася генералізована уреаплазменна інфекція, що викликало імунну відповідь у вигляді підвищення титрів специфічних антитіл до уреаплазм. Доведено розвиток системного запалення в більшості паренхіматозних органів (матка, печінка, легені, серце, щитоподібна залоза) з найбільш вираженими проявами у інфікованій нирці та легенях. Динаміка біохімічних показників відтворювала картину патогістологічних змін в нирках, сечовому міхурі, печінці, легенях, матці. U. parvum слід розглядати з одного боку як предиктора гострого запального процесу нирок й сечових шляхів, обумовленого класичними бактеріями, з іншого – має місце розвиток патологічних змін, які можуть розглядатись як первинний хронічний запальний процес, що має відмінності від класичного бактеріального.

Ключові слова: *експериментальний уреаплазменний пієлонефрит, уреаплазма, генералізована уреаплазменна інфекція.*

Introduction. Pyelonephritis which is infectious-inflammatory process of the kidney tissue is the most common kidney disease. Acute uncomplicated pyelonephritis (AUP) is the first stage of affecting the interstitium of a kidney. It is known that the most common pathogen of uncomplicated pyelonephritis is Escherichia coli (70-95% of cases); *Klebsiella pneumoniae, Proteus mirabilis and Staphylococcus spp.* are less common (Naboka, 2011; Sinyakova, 2013). In the 1980s the standard bacteriological study haven't revealed classical bacterial pathogens and in that time there were reports indicated patients with advanced «sterile pyelonephritis» had *Ureaplasma urealyticum*. Thus, according to the authors, *U.urealyticum* has been isolated from the bladder in monoculture or in association with other microorganisms in 75% of patients with reflux scarring and secretory function of the kidneys violation (Birch, Fairley, Pavillard, 1981). According to researchers *U.urealyticum* may be commensal in healthy man's bladder and have no pathological significance. At the same time, the authors did not rule out that ureaplasma can colonize previously damaged urinary tract or urinary tract of man with reduced resistance to infectious agents. Thus, the issue of *Ureaplasma spp.* and other mollicutes (*Mycoplasma hominis*) in the pathogenesis of urinary tract diseases remains open.

In our previous studies in patients with acute uncomplicated pyelonephritis using a complex microbiological diagnosis of biological material (culture-enzymatic and molecular genetic diagnostics) we found a high incidence of mollicutes in urinary (69.5%) and in the genital tract (67.5 %). Among mollicutes, ureaplasma was almost 2 time prevalent than mycoplasma, and in the urinary and genital tract equally (63.8% versus 35.8% and 62.7% vs. 31.7%, respectively). DNA amplification of ureaplasma has proven that *U.parvum* (biovar 1) prevailed – 50.4% of cases to 17.0% *U.urealyticum* (biovar 2) (Rudenko, 2017).

Thus, the data we obtained about the incidence of ureaplasma in the urinary and genital tract in patients with AUP on the one hand, and the lack of scientific information on the changes in the macroorganism that these pathogens may cause on the other, led us to create an experimental animal model.

The purpose of the study was to create an experimental model of pyelonephritis in vivo using biovar *Ureaplasma parvum*, which prevailed in patients with AUP, to study the histological picture of changes in kidney tissue and distal organs, biochemical and immunological parameters of the blood that characterize the response of the macroorganism to the development of the pathological process caused by this pathogen.

Materials and methods.

Methodological approaches to modeling in laboratory animals of human pathology are determined by the need to ensure, in the experimental conditions, of the maximum accurate reproduction of the pathological process, deprived of any side factors that affect the results of the experiment (Chereschnev, 2014). There are few methodological approaches among the experimental models of acute pyelonephritis (AP); though the vast majority of researchers created models using classical bacteria: *E.coli* (Hannan, 2016; Fedoruk, 2014), *S.saprophyticus* (Samodelkin, 2010), *S.epidermidis* (Vasilyev, 2001). There was only one case when *U.urealyticum* have been used for the modeling of pyelonephritis by administering ureaplasm in the ureter (Loran, 2008). It has been proved that infection of the upper urinary tract of experimental animals (rabbits) with *U.urealyticum* led to the development of primary chronic inflammatory process.

We performed an experiment on 10 female rabbits of the Chinchilla breed each weighing 2.5 kg in the age of 5 months, which were kept in common warrens and received water and standard feed in accordance with the recommendations for the maintenance. Four non-infected animals formed a control group for histological and biochemical studies.

For infecting animals we used the *U.parvum* strain isolated from the urine of patients with acute uncomplicated pyelonephritis in the Mycoplasma-DUO test system of BioMerieux (France) in conjunction with the polymerase chain reaction (PCR) using primers and equipment of the DNA firms «Technology», «Biocom» and «Amplisents» (Russia).

We created the pyelonephritis model by means of surgical intervention technique for rabbits under general anesthesia (thiopental sodium intravenously at a dose of 30 mg/kg: we sustained right-sided lumbotomy, isolated their right ureter and placed silk thread underneath with its both ends drawn and tied to the skin through the back muscles. Due to the ureter jamming to the muscles, its lumen was closed. At the end of this procedure, we administered in animal's renal duct, above compression site, 0.5 ml of suspension of *U.parvum* at a concentration of 10^5 CFU and after that sewed their wound. After 24 hours the ligature was cut through and pulled out of the skin.

The urine passage was restored after the removal of the ligature (Prat, 1958; Rudenko, 1985). We observed animals for 90 days studying their blood samples and biochemical and immunological indices dynamics. Serological studies were performed by means of standard method: *U.parvum* antigen as well as

neutralization reaction were used (Rudenko, 1985). Venous blood samples were examined before infectioning and thereafter on a monthly basis. To exclude the presence of classical bacteria we also seeded urine cultures each month using the cultural method of research. Culturally-enzymatic method was applied to isolate and identify ureaplasma.

Animals were withdrawn from the experiment by intravenous administration of lethal doses of thiopental sodium. During the autopsy we separated urine, blood and body samples for microbiological examination for the bacteria and ureaplasma presence. For histological studies, the tissue of the kidneys and other organs was fixed in 10% neutral formalin for 48 hours, after which they were de-mineralized in a riser battery of ethanol and placed in paraffin blocks. To prepare histological sections with a 5 microns thickness we used a septal microtome. The color of the sections was performed by hematoxylin-eosin.

The statistical processing of the obtained results was carried out by using standard methods of variation statistics, taking into account dissimilarities in the Student *t*-test, which was estimated using the confidence probability index (p), less than 0.05, with the program Statistica 6.0.

During the pilot study on laboratory animals, all bioethical rules and recommendations were implemented in accordance with the basic provisions of GLP (1981), the Convention of the Council of Europe on the Protection of Vertebrate Animals which are used for experimental and other scientific purposes, of 18.03.1986, the Order Ministry of Health of Ukraine No. 690 dated September 23, 2009 and the EEC No 609 dated 24.11.1986.

Results and discussion. Until the infectioning of healthy animals there weren't detected antibodies to ureaplasma in their blood (Fig. 1). The biochemical parameters of these blood serum samples were within normal limits and were used as controls (Table 1).

At 18 days after infection, serum antibodies appeared in the ureaplasma in the 1:8 titer (0.6 on the lg scale), reaching a maximum on 30th and 40th days of observation (1:32 and 1:64, which corresponded to 1,5 lg and 1.8 lg respectively) and gradually decreased by 90 days observation to 1:16, 1:32 (1.5-1.2 lg) (Fig. 1). Synthesis of antibodies to ureaplasma in animals infected by this pathogen indicates that there is a development of the inflammatory process caused by ureaplasma.

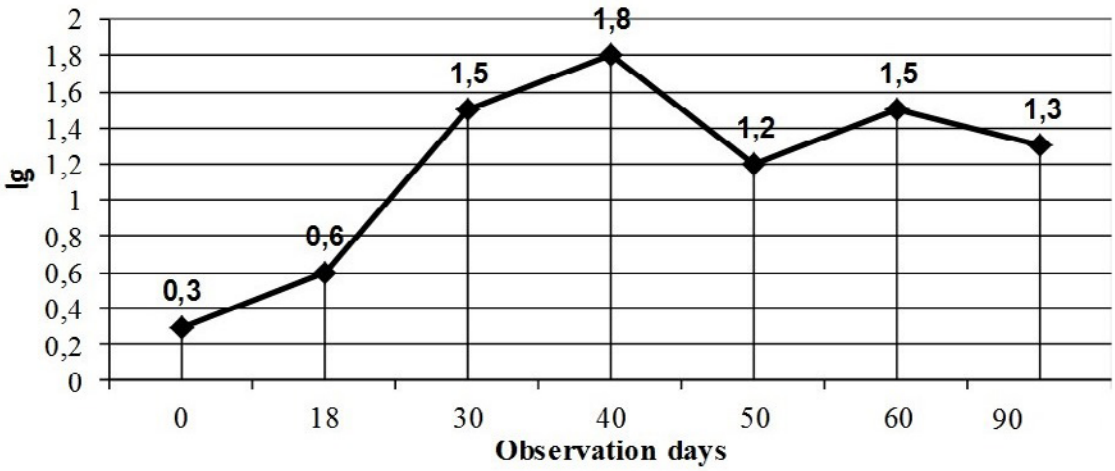


Fig. 1. Time course level of humoral antibodies titration according to the neutralization reaction in experimental animals

Regarding biochemical changes (Table 1), it was shown that at the 18th day of the animal experiment no significant changes in the parameters analyzed were found, but there was a tendency to their increasing. On 60th days in experimental animals we recorded changes in individual indices (creatinine primarily). Alanine aminotransferase (ALAT) level have also significantly increased. On 90 days indices were about to decrease, but levels of creatinine and ALAT remained high.

When creating the pyelonephritis model, we were most interested in the level of serum creatinine. Thus, the changes of the level of creatinine was as follows: an increase in the index was recorded on the 18th day of observation and reached its highest peak on 60th day, which is significantly exceeds the control value. This indicator can be affected by either violation of the microcirculation in the kidneys or the development of sclerotic changes as well as the violation of liver function, cardiovascular system and the development of pneumonia.

Table 1

Time course of biochemical indices of blood serum in experimental animals in follow-up observation

Indices	Control	Observation days		
		18	60	90
Creatinine, μmol/l	117,6±5,0	138,2±16,0	160,3±15,5 *	140,7±7,8 *
Urea, mmol/l	7,9±0,6	8,8±1,3	9,6±0,6	9,0 ± 0,64
Total protein, g/l	64,4±9,1	65,0 ± 2,0	72,0 ± 1,6	56,1 ± 5,2
Cholesterol, mmol/l	1,6±0,17	2,5 ± 0,24 *	1,2 ± 0,13	1,6 ± 0,23
Triglycerides, mmol/l	0,65±0,04	1,23 ± 0,27	nd	nd
Thymol test, SH, unit	0,43±0,13	0,81 ± 0,20	0,86 ± 0,23	0,30 ± 0,06
ALAT, IU/L	47,8±12,3	70,7 ± 10,9	123,4 ± 25,2 *	95,3 ± 17,1
ASAT, IU/L	40,2±5,6	60,2 ± 7,0	67,0 ± 8,6	41,7 ± 3,4

Notes: 1. p<0,05 – relative to data before infecting (*);
2. nd – not determined

Using thymol test we were able to confirm the development of the «syndrome of inflammation», which is accompanied by congestions and impaired blood flow in the liver. This is one of the most sensitive tests and even a slight increase in it indicates the development of the inflammatory process. Studying of aminotransferases (ALAT, ASAT) levels also suggests hepatocytes changed in the liver and the ALAT enzyme was released from the cells and got into the blood plasma. It should be emphasized that the «fast» thymol test rates at the time of withdrawal of animals from the experiment (90 days) decreased, while the level of ALAT remained high relative to animal control group.

Classical bacteria were not indicated by blood agar inoculation on blood samples, urine samples (urine sample was collected by using catheter before the animals were taken out from the experiment), as well as homogenates of the organs. In the «Mycoplasma-DUO» test system, *U.parvum* was detected in all internal organs, blood and urine with the highest concentration in liver, lungs, and infected kidneys.

In the Fig. 2-11 you can see the results of histological studies of organs of animals withdrawn from the experiment on 90 day after their infection. When studying the kidneys, bladder, uterus, liver, lungs, heart, thyroid gland no changes were found to characterize the presence of pathology (Fig. 2, 4, 6, 8, 10). Through histological study of organs biopsy of experimental animals infected by ureaplasma by its administration into the ureter, the definitive attribute was as such: lymphoid-cell infiltration with highest manifestation in the infected kidney, microcirculation loss with the erythrocytes prestase and stasis, interstitial edema and the development of sclerosis. We also provide photoregistration of histological studies of distinct animal organs infected with ureaplasma (Figures 3, 5, 7, 9, 11).

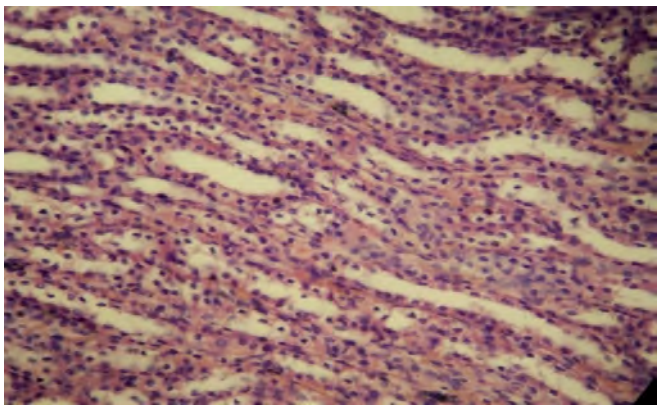


Fig. 2. The brain kidney substance – combined tubules in particular. The structure of the body is preserved (control). It was colored with hematoxylin-eosin* x 100
*** – for all histological preparations hereinafter**

Kidney. At the time of animal excretion from the experiment (after 90 days), the infected kidney cortical layer was marked by dystrophic changes of the proximal and distal tubules with a tendency for balloon dystrophy and necrobiosis of the epithelium; glomeruli were deformed (Fig. 3). In many tubules, especially

distal, there was a desquamation of the epithelium. The kidney stroma had florid lymphoid-cell infiltration and increased microvasculature blood filling, dilatation and parenchymal hemorrhage, focal sclerosis.

There were also pathological changes in the contralateral kidney in the form of increased microvasculature blood filling, edema, lymphoid-cell infiltration. Basically all of these changes were much less intense.

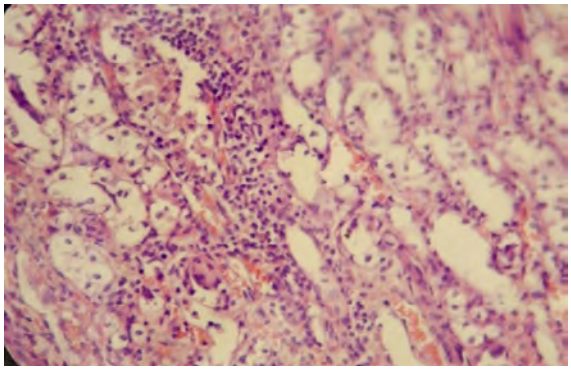
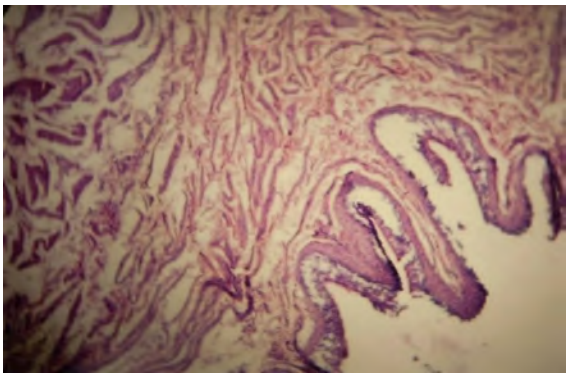


Fig. 3. Kidney cortical layer marked by dystrophic changes of the proximal and distal tubules with for a balloon dystrophy and necrobiosis of the epithelium in animals with experimental ureaplasmic pyelonephritis. There are also tubules with desquamated epithelium.The kidney stroma with florid lymphoid-cell infiltration and increased microvasculature blood filling. x 100

Fig. 4. The urinary bladder wall (control). The structure is preserved. Urothelium with minor changes, desquamated sometimes. x 100



Urinary bladder. The surface epithelium of the bladder of experimental animals which were infected by *U.parvum* was marked by dystrophic changes; we also observed desquamation in some places. Lamina propria with a sharp edema. Muscle layers were also associated with the symptoms of severe edema and sclerosis (Fig. 5).

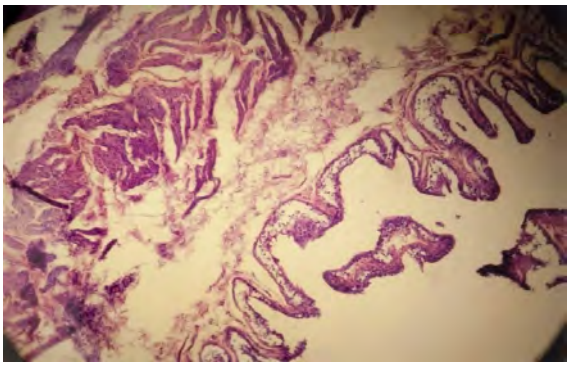


Fig. 5. The urinary bladder of an infected animal. Extreme dystrophic changes of the surface epithelium with desquamation. Lamina propria sharp edema, muscular layer swelling, initial sclerosis.
Scaling – 100

Uterus. Female rabbits had their parametrium sharply increased in blood filling (erythrocyte stasis) and edema (Fig. 7).

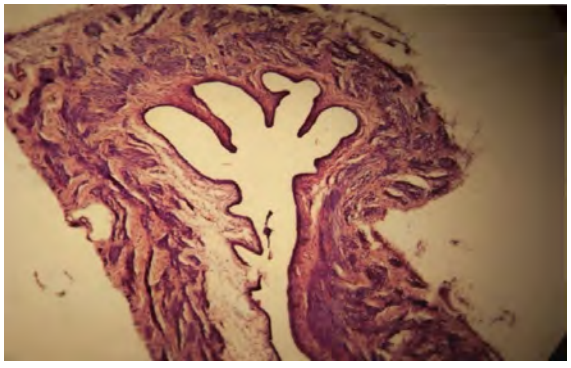


Fig. 6. Uterus (control). Endometrium and myometrium without changes. The structure is preserved. x 100

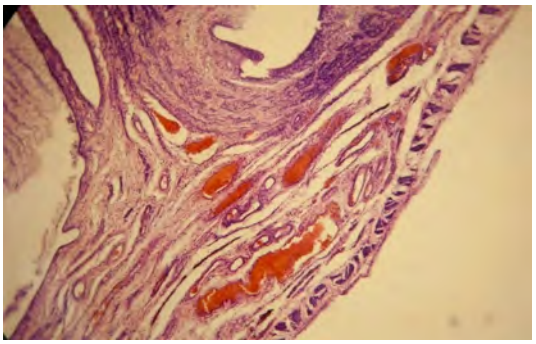


Fig. 7. Infected animal uterus. Sharp increase in blood filling and parametrium edema. x 400

Liver. In the liver we observed hepatocyte discomplexation, space of Disse enlargement, increased blood filling (erythrocyte stasis), edema and florid lymphoid-cell infiltration in the area of the triad (Fig. 9).

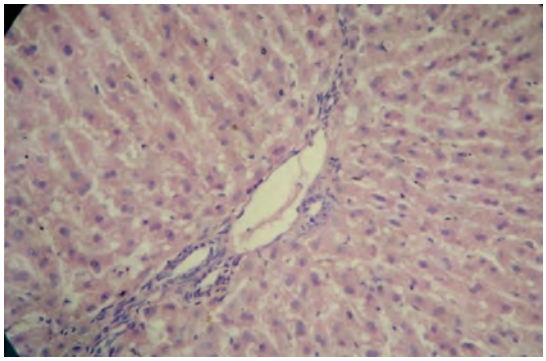


Fig. 8. Liver (control). There are no inflammatory changes. The structure is preserved. x 100

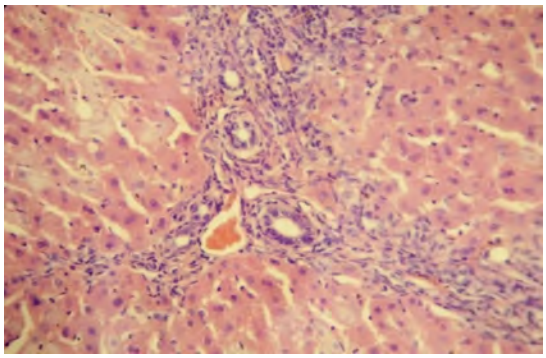


Fig. 9. Infected animal liver: hepatocyte discomplexation, Disse's space enlargement with symptoms of severe lymphoid-cell infiltration in the area of the triad, increased blood filling. x 100

Lungs. In the lung tissue of infected animals, there was a sharp increase in blood filling, a distinct erythrocyte stasis, interalveolar membranes thickening. At the same time, there was a lymphoid-cell perivascular and perialveolar infiltration, indicating the development of the inflammatory process (pneumonia). We also observed arteries sclerosis of different caliber (Fig. 11). Lungs parenchyma was usually of lowered pneumatization due to filling the of the alveolus lumen with erythrocytes.

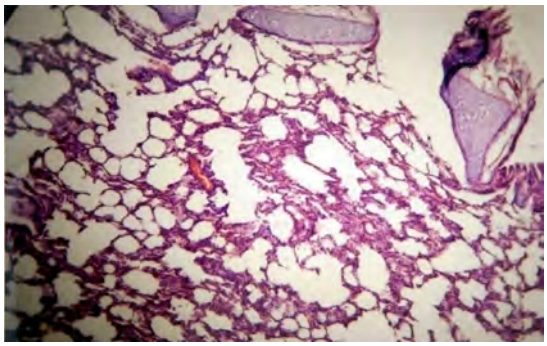


Fig. 10. Lungs (control). Alveolus, usual thickness walls. x 100

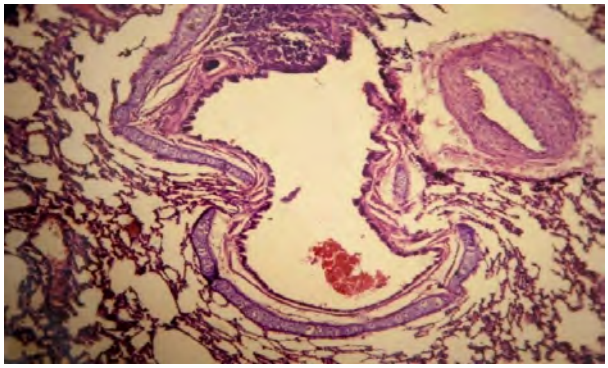


Fig. 11. Infected animal lungs with sharply increased vascular blood filling, thickening of interalveolar membranes with florid erythrocytes stasis. Pneumatization is reduced due to the filling of the lumen of the alveolus by the erythrocytes mass. x 100

Thus, the data we obtained when creating pyelonephritis model in female rabbits using one of the most frequently detectable pathogens in patients with acute uncomplicated pyelonephritis – *U.parvum*, allowed us to state that animals infected with ureaplasma have hematogenous dissemination of the pathogen in various organs. It is also clear that patients have generalized inflammatory process caused by lymphoid-cell infiltration as an evidence of the results of histological studies. The development of ureaplasmosis in all infected animals was further proved with an increasing of the antibodies titres to ureaplasma and the biochemical changes in blood time course. Systematic affecting of the organs has specific pathological features amidst subacute clinical implications of pyelonephritis with a tendency for sclerotic degeneration of interstitium and the development of initial angiosclerosis.

Clinical and experimental data on the role of ureaplasma, namely *U.parvum*, in the pathology of humans and animals we obtained should be considered as a predictor of acute inflammation of a kidney and urinary tract caused by classical bacteria on the one hand and a development of pathological changes that can be considered as a primary chronic inflammatory process, which differs from the bacterial and is accompanied by a stasis, a redness of the erythrocytes, edema, lymphoid-cellular infiltration and the initial sclerosis of organs on the other. It should be specifically noted that these pathogens, reaching other organs using bloodstream, cause analogous changes. Obtained data confirm our hypothesis on the role of mollicutes in the pathogenesis of acute uncomplicated pyelonephritis and make us re-examine our understanding of their role in multi-vector pathology.

Conclusion

1. The use of *U.parvum* in the development of an experimental model of acute pyelonephritis in rabbits leads us to development of lymphoid-cell infiltration in the kidneys, microcirculation disorders (erythrocytes stasis and prestase), stroke edema, initial symptoms of sclerosis, interstitia and angiosclerosis, dystrophic changes in the epithelium of the renal tubular system and urothelium of the urinary bladder.

2. We proved the development of systemic inflammation in the greater part of parenchymal organs (uterus, liver, lungs, heart, thyroid gland) in the form of stromal edema, lymphoid-cellular infiltration, microcirculation disorders, sclerotic and dystrophic changes in parenchymal organs.

3. The use of *U.parvum* in the development of a model of pyelonephritis in animals causes an immune response in the form of increasing specific antibodies titres to ureaplasma reaching a maximum (1:32 and 1:64) on 30-40 observation days.

4. It has been experimentally proved that *U.parvum* biovar, which specific weight among mollicutes is 70%, can be categorized to conditionally pathogenic microorganisms that can create a background for the infectioning of the kidneys with classical bacteria and cause the development of the infectious and inflammatory processes. Therefore, we believe such a background is favorable for bacterial flora, where even bacteria with a limited spectrum of pathogenic properties can cause the development of acute pyelonephritis.

5. The above-mentioned histopathological changes in the infected kidney and interstitium edema, microcirculation of the cortical layer loss and necrobiotic changes in the epithelium of the tubular structures of the contralateral kidney also create conditions for the development of chronic renal insufficiency in experimental animals.

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