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Research Article

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Pilot testing for long-term impact of glycerol-induced acute kidney injury on oxalate homeostasis in rats

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Abstract. *There is a general lack of research on the long-term effects of acute kidney injury (AKI) on oxalate-degrading bacteria (ODB) and their total oxalate-degrading activity (ODA) in fecal microbiota. In the present pilot study, we separately evaluated the changes in the ODB number and their total ODA in fecal microbiota at 3-time points after glycerol-induced AKI. In addition, we assessed the interactions between AKI-induced renal histopathological changes and ODB, total fecal ODA, and plasma and urine oxalate concentrations in rats.*

Methods. *The male Wistar rats (200-300 g, n = 20) on oxalate-free diet were randomly divided into 2 groups. After 24-h of water deprivation, experimental group 1 (n = 10) received an intramuscular injection of 50% glycerol (10 ml/kg of body weight), and group 2 (n = 10) served as a control. The numbers of ODB (incubated in a highly selective Oxalate Medium and determined using the culture method), total fecal ODA and urinary oxalate (UOx) excretion were measured after injection on days 8, 22 and 70. The method of redoximetric titration with a KMnO₄ solution was adopted to evaluate total ODA in fecal microbiota. Renal injury was assessed by histopathology examination, serum creatinine plasma oxalic acid (POx) concentration and daily proteinuria levels after removing the animals from the experiment on day 70.*

Results. *After glycerol injection on days 8 and 22, no differences were found in the numbers of ODB, their total fecal ODA, and UOx excretion level between the experimental and control groups. However, after AKI initiation on day 70, the numbers of ODB, total fecal ODA, and daily UOx excretion were significantly lower in the experimental group as compared with the control group. In addition, in 10 weeks following AKI, the number of ODB had a direct correlation with UOx excretion and an inverse correlation with POx and serum creatinine concentrations and daily proteinuria. Total ODA in fecal microbiota was directly associated with the percentage of renal interstitial fibrosis and the average glomerular volumes in the experimental rats.*

Conclusions: *AKI had long-term negative effects on the quantitative and qualitative characteristics of ODB in fecal microbiota in rats. Moreover, the results of our study confirmed an increasing trend in total fecal ODA according to the aggravation of renal interstitial fibrosis and glomerular volume in rats' kidneys. Further studies are warranted to gain more insight into the mechanism of oxalate homeostasis impairment in AKI.*

Key words: acute kidney injury, oxalate, gut microbiota, rats.

Conflict of interest statement. The authors declare no competing interest.

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Пілотне дослідження довгострокового впливу гліцерин-індукованого гострого пошкодження нирок на гомеостаз оксалатів у щурів

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Резюме. У цьому пілотному дослідженні ми оцінили динаміку кількості оксалатдеградувальних бактерій (ОДБ) та їх загальної оксалатдеградувальної активності (ОДА) у фекальній мікробіоті щурів на 8, 22 та 70 дні після гліцерин-індукованого гострого пошкодження нирок (ГПН). Крім того, ми проаналізували взаємозв'язок між гістопатологічними змінами нирок, ОДБ, загальною фекальною ОДА, концентраціями оксалатів у плазмі та сечі щурів.

Методи. Самці щурів лінії Вістар (200-300 г, n = 20) випадковим чином були розподілені на 2 групи. Після 24-годинної водної депривації, експериментальній групі щурів (n = 10) внутрішньом'язово введено 50 % гліцерин (10 мл/кг маси тіла), 2 група тварин (n = 10) була збережена у якості контролю. Кількість ОДБ (інкубованих у високоселективному оксалатному середовищі та визначених культуральним методом), загальну ОДА та екскрецію оксалату з сечею досліджували на 8, 22 та 70 дні після ін'єкції гліцерину. Метод редоксиметричного титрування з КМпО4 було адаптовано для оцінки загальної ОДА в мікробіоті фекалій. Ураження нирок оцінювали за допомогою гістопатологічного дослідження; концентрації сироваткового креатиніну, оксалової кислоти плазми та добової протеїнурії досліджували після вилучення тварин з експерименту на 70-й день.

Результати. Після ін'єкції гліцерину на 8 і 22 дні, нами не визначено відмінностей у кількості ОДБ, їх загальної ОДА у фекальній мікробіоті та екскреції оксалату між дослідною та контрольною групами. Однак через 10 тижнів після ініціації ГПН, кількість ОДБ, їх загальна ОДА та добова екскреція оксалатів була значно нижчою в експериментальній групі порівняно з контролем. Крім того, через 10 тижнів після ініціації ГПН кількість ОДБ мала прямий кореляційний зв'язок з оксалурією та зворотній зв'язок з концентраціями оксалової кислоти та креатиніну крові, добовою протеїнурією. Загальна ОДА в мікробіоті фекалій була прямо асоційована з відсотком ниркового інтерстиціального фіброзу та середнім гломерулярним об'ємом експериментальних щурів.

Висновки. ГПН мало довготривалий негативний вплив на кількісні та якісні характеристики ОДБ у фекальній мікробіоті щурів. Більше того, результати нашого дослідження продемонстрували зростання загальної фекальної ОДА відповідно до збільшення інтерстиціального фіброзу та середнього гломерулярного об'єму у нирках щурів. Для кращого розуміння механізму порушення гомеостазу оксалату за ГПН необхідні подальші дослідження.

Ключові слова: гостре ураження нирок, оксалат, мікробіота кишечника, щури.

Introduction. Cross-talk between gut microbiota and oxalate homeostasis is currently being actively discussed in scientific literature and has become the basis of global efforts to reduce the formation and progression of oxalate kidney stones [1-4]. Emerging evidence suggests that many gut bacteria (*Oxalobacter formigenes*, *Enterococcus* spp., *Lactobacillus* spp., *Bifidobacterium* spp., *Bacillus* spp) have the oxalate-degrading ability and points to their crucial role in oxalate homeostasis [1, 5-7]. It has been demonstrated that intestinal oxalate-degrading bacteria (ODB) could provide a complementary route of oxalate excretion that becomes more evident when kidney function declines [3-5, 8-10]. In

chronic kidney disease (CKD) patients, dietary restriction, uremia, and metabolic acidosis are the main factors affecting quantitative and qualitative characteristics of gut microbiota composition that contribute to impaired oxalate homeostasis and, as a consequence, hyperoxaluria and hyperoxalemia conditions [2, 5, 11-14]. However, although CKD-associated gut microbiota alterations are highlighted in multiple recent publications [15-17], there is little evidence of the impact of CKD on the number and functional capacity of ODB. In our previous reports, we have demonstrated that oxalate homeostasis in CKD patients was influenced not so much by the quantity of ODB in the intestinal microbiota but by the total ability of different strains of ODB to metabolize oxalate [7, 18-20].

Furthermore, in a rat model of antibiotic-induced gut dysbiosis, we have observed a significant growth in the ODB number on the 1-st day following the treatment withdrawal, and despite the increase in the ODB quantity, a substantial reduction in total fecal oxalate-degrading activity (ODA) compared to control groups

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was noted [20]. Bacterial ODA in fecal microbiota rather than their quantity was associated with urinary oxalate (UOx) excretion and plasma oxalate (POx) concentration. These data provided preliminary evidence on the crucial role of ODA rather than ODB number in oxalate homeostasis in both CKD-related and antibiotic-induced dysbiotic conditions. However, to the best of our knowledge, there is a general lack of studies on the impact of acute kidney injury (AKI) on the ODB number and the total ODA in fecal microbiota.

Oxalate nephropathy due to increased dietary oxalate intake or enteric hyperoxaluria is a well-known cause of AKI [21-23]. Oxalate crystal deposition in kidney tubules and/or the interstitium leads to acute tubular injury and interstitial infiltration resulting in AKI and interstitial fibrosis [21-23]. However, whether AKI per se can trigger intestinal oxalate handling and disrupt oxalate homeostasis remains unknown.

Therefore, in the present pilot study, we separately evaluated the changes in the ODB number and their total ODA in fecal microbiota at 3-time points after glycerol-induced AKI. In addition, we assessed the interactions between AKI-induced renal histopathological changes and ODB, total fecal ODA, and plasma and urine oxalate concentrations in rats.

Materials and Methods. Animal Care and Use. The male Wistar rats (200-300 g, n = 20) were bred and housed in the conventional animal facility of the ESC "Institute of Biology and Medicine" of Taras Shevchenko National University of Kyiv (Kyiv, Ukraine) under standard environmental conditions. All the rats were kept in a standard cage and had free access to a standard stock diet and tap water provided ad libitum. To ensure standardized gut microbiota, rats from all groups were kept in the same room and maintained by the same person. The "Guide for the Care and Use of Laboratory Animals" (National Research Council 2011) was followed. The procedures used and the care of animals were approved by the animal committee of the ESC "Institute of Biology and Medicine" of Taras Shevchenko National University of Kyiv (Protocol # 18/01/2020).

Experiment design and outline. Animals were numbered and randomly divided into 2 groups. After 24-h of water deprivation, the rats of Group 1 (n = 10) received an intramuscular injection of 50% glycerol (10 ml/kg of body weight), and Group 2 (n = 10) served as a control group.

On day 8 (in a week) after glycerol injection, the amount of ODB and their total ODA in fecal microbiota, as well as daily urinary oxalate (UOx) excretion and proteinuria levels were measured in each group of rats. Then we repeated the same tests on day 22 (in 3 weeks) after AKI initiation. On day 70 (in 10 weeks) of the experimental period, all rats were anesthetized with urethane (1.1 g/kg, i.p. Sigma-Aldrich, Germany). Blood samples were collected by cardiac puncture immediately after death, and in addition to the aforementioned markers in the rats' feces and urine, plasma oxalic acid

(POx) and serum creatinine were determined. Finally, after removing the animals from the experiment on day 70, both kidneys were fixed in 10% neutral buffered formalin (Thermo Fisher Scientific, USA) for histological studies.

ODB and total ODA analysis in the rats' fecal samples. Rats' fecal samples (1g) were collected and immediately dispersed in 9 ml highly selective media Oxalate Medium as described previously [7, 20]. In brief, for the determination of ODB number, serial dilutions (10-3, 10-5, 10-7) of each dispersed sample were prepared. One aliquot of 0.1 ml of each dilution was seeded by a pure plate technique and cultured anaerobically (BD BBL™ CO2 gas generators, BioMerieux, France) at 37°C for 5 days on a solid sterile Oxalate Medium. Finally, we determined the quantitative composition of microorganisms, which grew as single colonies. The number of fecal ODB was calculated as lg of colony-forming unit per 1 g of feces (lg CFU/g).

For the determination of the ODA in feces, we transferred 5 ml of dispersed feces samples to 45 ml Oxalate Medium and kept it under anaerobic conditions for 48 hours at 37°C in tightly closed 50 ml bickers (test solution). The method of redoximetric titration with a KMnO4 solution was adopted to evaluate total ODA in fecal microbiota [7, 20]. The results were expressed in % degradation of sodium oxalate per 0.01 g of feces for rat fecal specimens.

Blood and urine measurements. After sample collection, the blood tubes were delivered to the Laboratory and centrifugated at 2000 X g for 15 minutes at room temperature. After centrifugation, the plasma was separated into 1.5 ml Eppendorf tubes labeled with the animals' numbers and analyzed immediately. POx concentration was measured spectrophotometrically using a commercially available kit (MAK315, Sigma, Barcelona, Spain) according to the manufacturer's protocols. Rats' 24-h urine samples were collected in individual metabolic cages and delivered to the Laboratory immediately. Daily UOx excretion was determined using an oxalate oxidase/peroxidase reagent (BioSystems, Barcelona, Spain). Serum creatinine and urinary protein levels were additionally measured using Flexor Junior Analyzer.

Histological examination. The kidneys were fixed in 10% buffered formalin solution, dehydrated, cleared and embedded in paraffin (Histowax, Leica, Germany). Blocks were cut into 4-µm-thick paraffin sections stained with hematoxylin and eosin (Biognost, Croatia), periodic acid-Schiff (PAS, Richard-Allan Scientific, USA), and picro sirius red (Sigma-Aldrich, USA). The obtained histological sections were examined and photomicrographed using an image analysis system based on the Olympus BX51 microscope with Olympus DP-Soft 3.2 software (Japan). Measurements were performed on digital micrographs obtained at a magnification of x200.

The histological examination included the kidneys of rats of both the experimental and control

groups obtained at the 1st (designated as experimental model control) and the 10th week after AKI. Signs of acute and chronic damage to the glomerular, tubular, interstitial, and vascular compartments of the kidney were evaluated. Glomerular volume was determined using the stereometric method [24]. Cortical interstitial fibrosis was measured by computerized image analysis on sections stained with picrosirius red [25, 26].

Statistical analysis. Data analysis and all graphs were performed using the MedCalc Statistica Software version 20.011 (Ostend, Belgium). The average means (M) and the standard deviations (SD) or the median (Me) and the interquartile ranges (Q25-Q75) were calculated according to a distribution. For the statistical analysis, we used the Student's t-test and the non-

parametric (U-test) Mann-Whitney or Kruskal-Wallis tests with the Conover test for post-hoc comparisons, respectively. The Spearman's test was used for the correlation analysis. Two-factor analysis with repeated measures ANOVA was performed followed by Turkey post-hoc test for multiple comparisons.

Results. Long-term effects of glycerol-induced AKI on intestinal ODB and oxalate homeostasis in rats. After glycerol injection on days 8 and 22, no differences were found in the numbers of ODB, their total fecal ODA, and UOx excretion level between the experimental and control groups. However, after AKI initiation on day 70, the numbers of ODB, total fecal ODA, and daily UOx excretion were significantly lower in the experimental group as compared with the control group (Fig. 1).

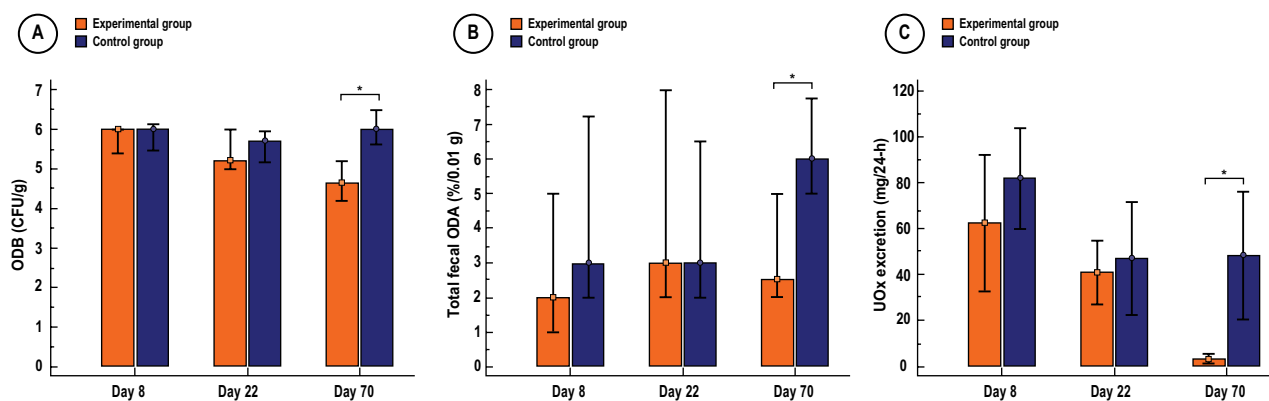


Fig. 1. Glycerol-induced AKI triggered changes in the ODB number and their ODA in rats' feces and UOx excretion.

The data are presented as Me (Q25-Q75) and compared using the two-factor repeated ANOVA analysis.

(A): Changes in ODB number in the rats' fecal microbiota during the experimental period; (*): $P < 0.001$.

(B): Changes in total fecal ODA in rats during the experimental period.

(C): Changes in UOx excretion during the experimental period; (*): $P < 0.001$.

Abbreviations: ODA, oxalate-degrading activity; ODB, oxalate-degrading bacteria; UOx, urinary oxalate.

Interestingly, we observed a gradually decreasing trend in the ODB number and UOx excretion level in the rats of the experimental group according to the weeks of the study period (Fig. 2).

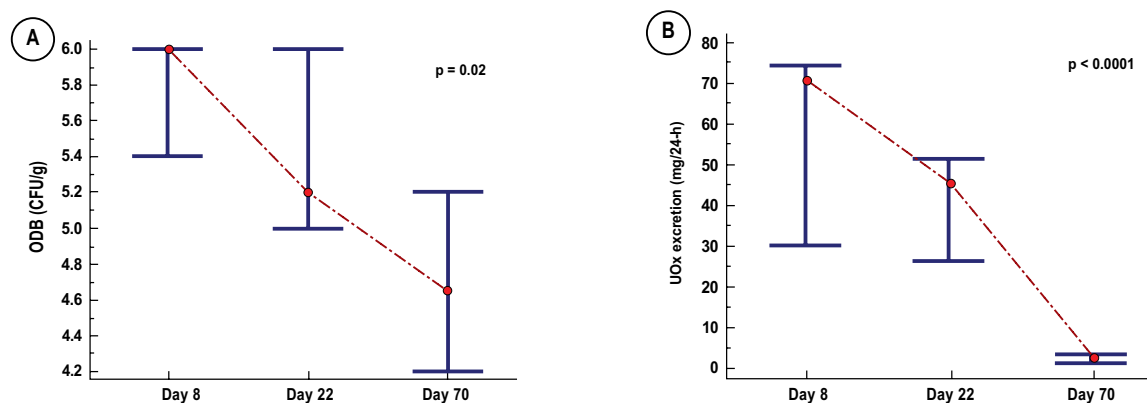


Fig. 2. A decreasing trend in the ODB number (A) and UOx excretion level (B) in the rats of the experimental group according to the weeks of the study period.

The data are presented as Me (Q25-Q75) and compared using the two-factor repeated ANOVA analysis. Abbreviations: ODA, oxalate-degrading activity; ODB, oxalate-degrading bacteria; UOx, urinary oxalate.

Conversely, both serum creatinine and POx concentrations measured on day 70 were significantly higher in the rats of the experimental group compared to the

control group (Fig. 3). The higher serum creatinine was the higher POx concentration occurred ($r = 0.72$, $p = 0.0007$).

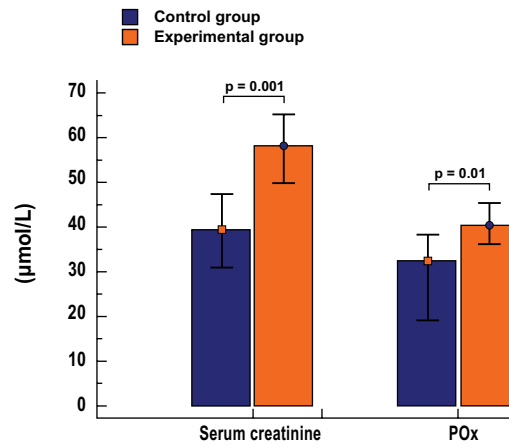


Fig. 3. Serum creatinine and POx concentrations on day 70 in the experimental rats compared to the control group. The data are presented as Me (Q25-Q75) and compared using the Kruskal-Wallis test. Abbreviation: POx, plasma oxalate.

We did not find any association between the number of ODB and their ODA at any examined time-points. Moreover, ODA in the rats' fecal microbiota was not associated either with plasma and urine oxalate or other determined markers. In contrast to ODA, the

number of ODB on day 70 had a direct correlation with UOx excretion and an inverse correlation with POx and serum creatinine concentrations, and daily proteinuria (Fig. 4).

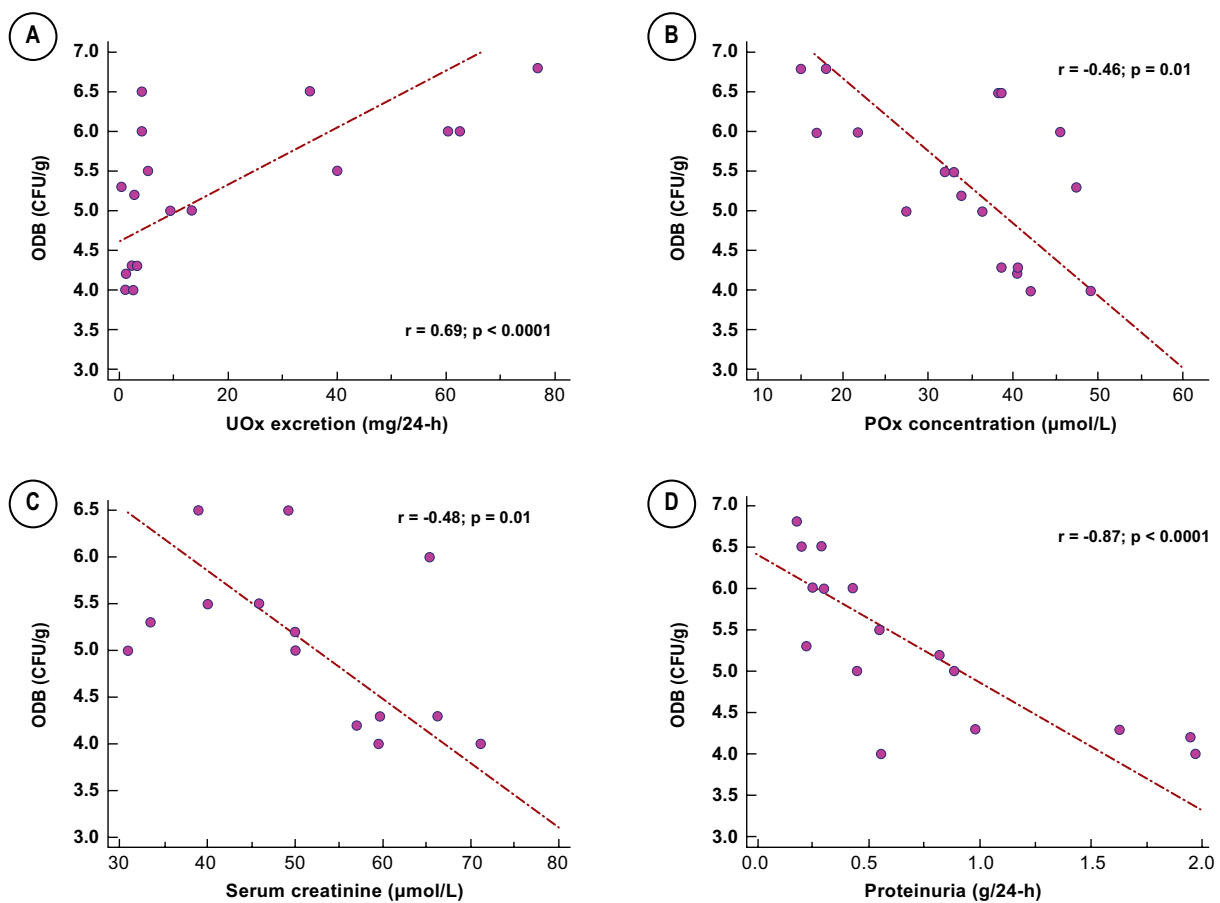


Fig. 4. The association between the number of ODB in the rats' fecal microbiota and UOx excretion (A), POx concentration (B), serum creatinine (C), and proteinuria (D) level. Abbreviation: ODA, oxalate-degrading activity; ODB, oxalate-degrading bacteria; POx, plasma oxalate; UOx, urinary oxalate.

Association between kidney histopathological changes and oxalate homeostasis in rats with glycerol-induced AKI. On day 8 after glycerol injection, histopathological changes in the rats' kidneys were characterized by acute tubular necrosis with moderate inflammatory response, the presence of pigment

casts, and early ischemic changes (Fig. 5A). The histopathological changes on day 70, were characterized by a small increase in glomerular size, slight chronic tubulointerstitial changes, and predominant recovery of renal cytoarchitectonics after acute tubular injury (Fig. 5B, C).

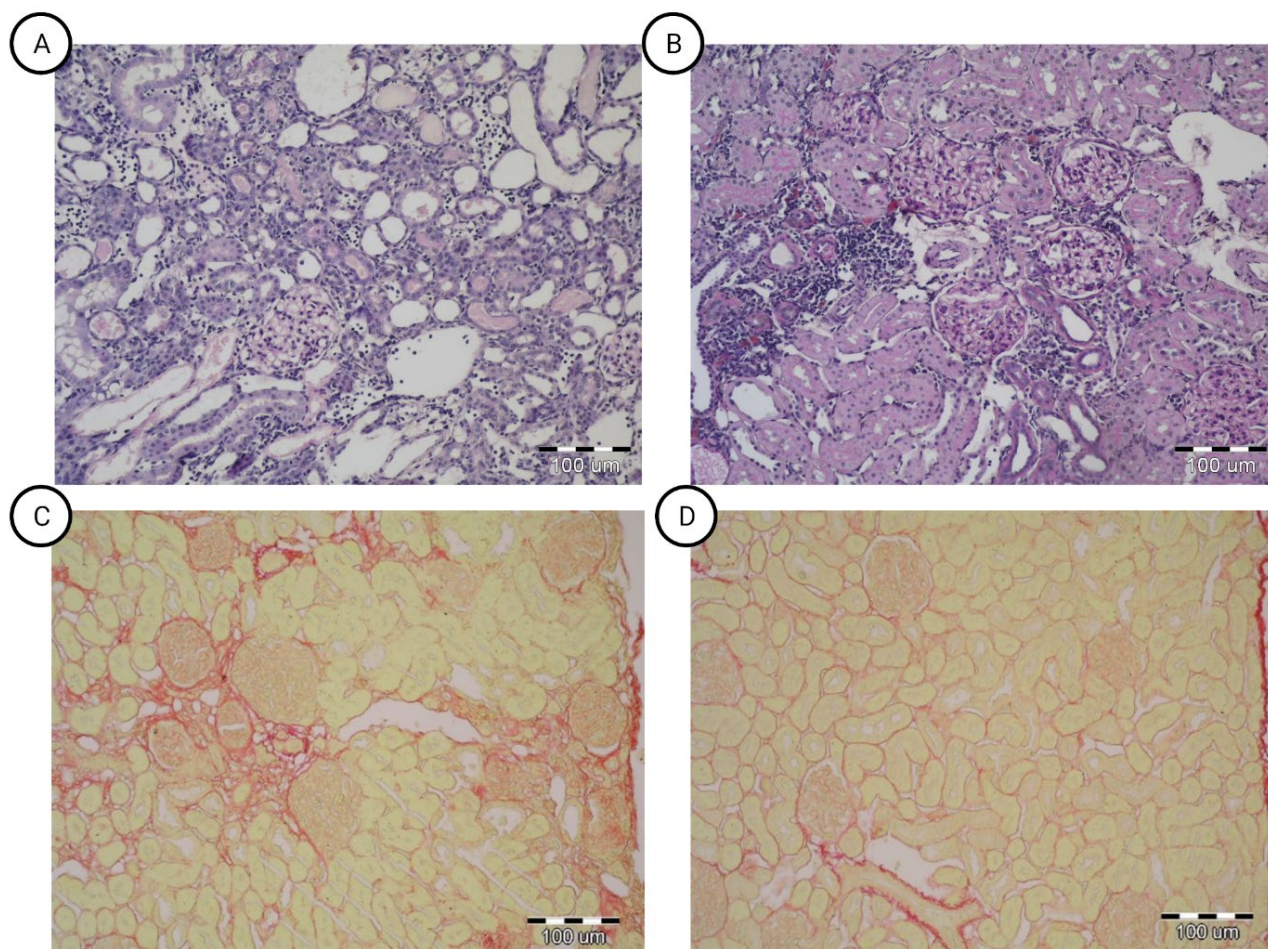


Fig. 5. Histopathological changes in the rats' kidneys on days 8 (A) and 70 (B, C after glycerol-induced AKI), and control (D).

(A) Cortical labyrinth of a rat's kidney on day 8. Tubular luminal dilatation and simplification of the lining epithelium and loss of the brush border in the proximal convoluted tubules and amorphous luminal debris in the distal tubules, and severe peritubular capillaritis.

(B) Cortical labyrinth of a rat's kidney day 70. Single glomeruli with a thickening of the Bowman capsule. Among the cellular infiltrate there are groups of cells that contain brown pigment. Foci of tubular atrophy associated with mononuclear infiltration. PAS.

(C) Cortical labyrinth of a rat's kidney day 70 and (D) control. Single glomeruli with markedly enlarged. Small subcapsular and radial scars with atrophy of some tubules. Picro Sirius red.

All digital images $\times 200$

Average glomerular volume on day 8 after glycerol-induced AKI in the experimental group was 34% lower than in the control rats ($3.84 \pm 0.42 \times 10^5 \mu\text{m}^3$ vs $5.83 \pm 0.34 \times 10^5 \mu\text{m}^3$, respectively, $p = 0.036$). In contrast, the average glomerular volume in the experimental group on day 70 showed a 15% increase compared to the control group ($6.71 \pm 0.95 \times 10^5 \mu\text{m}^3$,

$p = 0.043$) (Fig. 6A). The fraction of cortical Sirius-positive staining in the experimental group on day 8 after glycerol-induced AKI did not differ from the control ($12.8 \pm 0.8\%$ vs $12.6 \pm 0.6\%$, respectively, $p = 0.76$). However, on day 70, a 24% increase in interstitial fibrosis ($15.6 \pm 0.6\%$) was observed compared to the control rats (Fig. 6B).

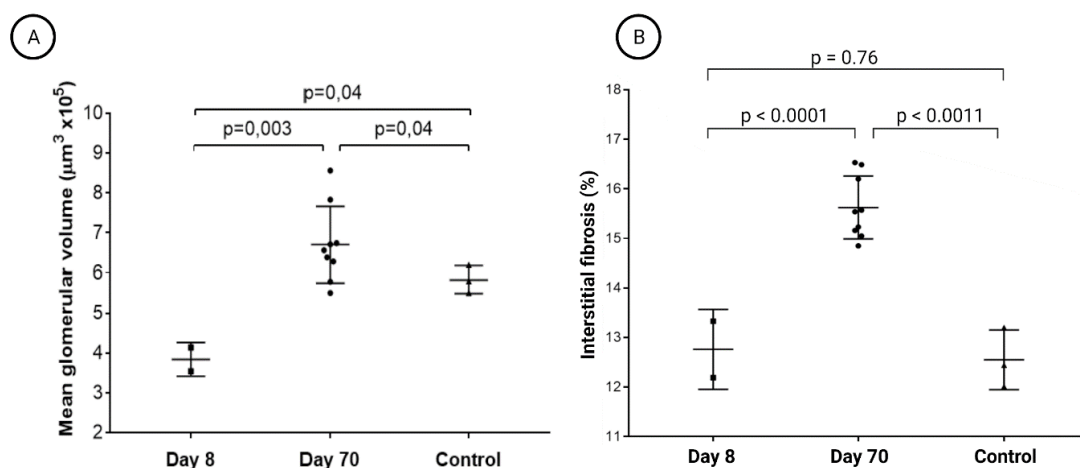


Fig. 6. The average glomerular volume (A) and interstitial fibrosis level (B) in the experimental group compared to the control group on day 70. The data are presented as M ± SD and compared using the Student's t-test.

Correlation analysis demonstrated a direct association of total fecal ODA with the percentage of renal interstitial fibrosis and the average glomerular volumes in

a rat of the experimental group on day 70 (Fig. 7). The higher the average glomerular volume was, the higher the POx concentration was observed ($r = 0.63, p = 0.04$).

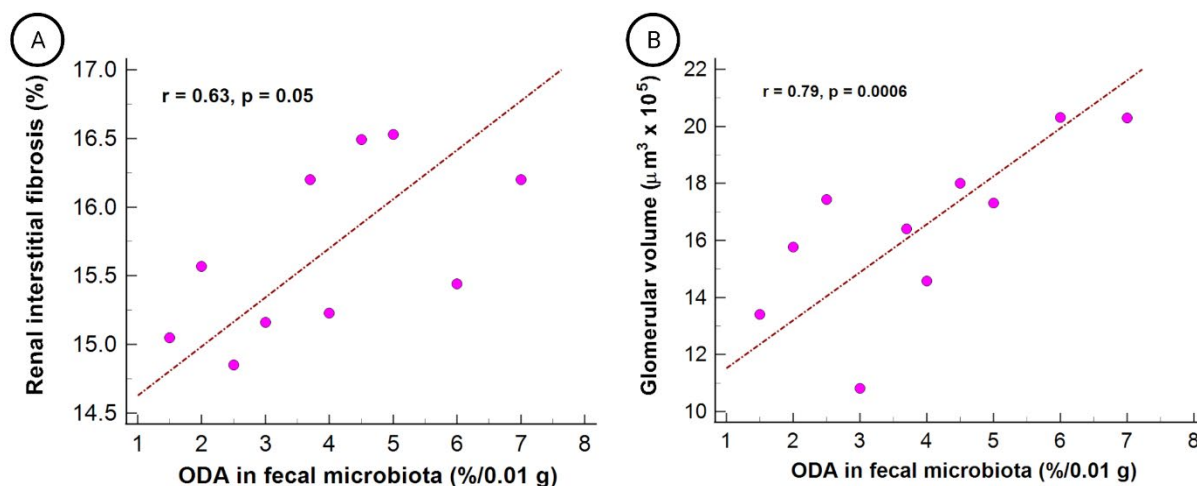


Fig. 7. Association of total fecal ODA with the percentage of renal interstitial fibrosis (A) and the average glomerular volumes (B) in the rats with glycerol-induced AKI.

Discussion. Since similar microbiota patterns contribute to oxalate degradation and AKI severity [3, 27-30], we speculate that AKI could violate oxalate homeostasis by decreasing ODB number and their ODA in intestinal microbiota. Therefore, in the present study, we tested the hypothesis that similar to the CKD-related pathway, glycerol-induced AKI provokes oxalate homeostasis impairment and might be influenced not so much by the quantity of ODB in the intestine microbiota as by the total ability of ODB to degrade oxalate. To this end, we separately evaluated the changes in the ODB number and their total ODA in fecal microbiota at 3-time points after glycerol injection. In addition, we assessed the interaction between kidney histopathological changes, ODB, total fecal ODA, and plasma and urine oxalate concentrations in rats.

There are some new findings in the present study. First, glycerol-induced AKI resulted in a significant decrease in UOx excretion, ODB number and total ODA in the rats' fecal microbiota after 10 weeks. Notably, the ODB number and UOx excretion level had a gradually decreasing tendency according to the weeks of the study period. Second, in 10 weeks following AKI, the number of ODB had a direct correlation with UOx excretion and an inverse correlation with POx and serum creatinine concentrations and daily proteinuria. Third, total ODA in fecal microbiota was directly associated with the percentage of renal interstitial fibrosis and the average glomerular volumes in the experimental rats.

Current studies highlight the gut-kidney cross-talk in AKI and indicate a bidirectional interaction between gut microbiota and acute kidney function de-

cline [27-29]. A great example of this interaction has been recently shown in a mouse model of kidney ischemia/reperfusion injury (IRI) by Yang et al [29]. The authors have identified a relative increase of *Escherichia*, *Enterobacter*, and a decrease of *Lactobacillus*, *Ruminococcaceae*, *Faecalibacterium*, and *Lachnospiraceae* within the first 24 hours and only partial restoration of the species richness in 7 days following kidney injury [29]. These changes in the microbial community were associated with decreased levels of short-chain fatty acids, intestinal inflammation and leaky gut resulting in enhanced kidney dysfunction. Moreover, the authors have demonstrated that dysbiotic microbiota transferred to germ-free mice aggravated kidney injury while the antibiotic-induced depletion of gut microbiota before kidney injury had a significant renoprotective effect against IRI [29]. In a more recent study, Yang et al. have revealed that pretreatment with *Bifidobacterium bifidum* BGN4 for 2 weeks before IRI significantly decreased AKI-induced dysbiosis and the severity of kidney damage through immunomodulation effects of the probiotic [31]. It should be emphasized that all aforementioned studies were limited to a short-term period of observation and there is a general lack of research on the long-term effects of AKI on intestinal microbiota composition. Unfortunately, the design of the present study did not include the determination of ODB and their ODA on the first day after AKI initiation. However, in agreement with the above studies, we found no difference in the number of ODB following a week and 3 weeks of the experimental period which can be explained by partial restoration of the bacteria richness in 7 days following kidney injury [29].

According to our previous reports, ODA in fecal microbiota plays a more obvious role than the ODB number per se in oxalate homeostasis in both CKD-related and antibiotic-induced dysbiotic conditions [7, 20]. In line with the aforementioned studies, total fecal ODA but not ODB number was associated with the percentage of interstitial fibrosis and glomerular volume in the rats' kidneys. Glomerular volume is a well-known parameter of kidney structure that determines the filtration surface area and correlates with the glomerular filtration rate [32, 33]. It has been shown that a decrease in glomerular volume is a sign of ischemic kidney damage while glomerular hypertrophy is a strong predictor of glomerulosclerosis [32, 33]. The glomerular volume results obtained here were consistent with the experimental period and indicated partial glomerulosclerosis in the rats' kidneys 10 weeks after glycerol-induced AKI. In our opinion, AKI to chronic kidney disease transition is the main cause that triggers alternative pathways for oxalate processing and, consequently, increased ODA in fecal microbiota.

The present study has several limitations. First of all, glycerol-induced AKI is the most frequently applied model for studying AKI which induces rhabdomyolysis. However, between 15% and 30% of all AKI cases can be attributed to rhabdomyolysis [34]. Thus, we cannot thoroughly conclude that the obtained results may reflect changes in oxalate balance in AKI of another genesis. Second, ODB and ODA testing on day 1 following glycerol-induced injury could have strengthened our claims of oxalate homeostasis in AKI. However, despite these limitations, our study is the first to demonstrate a direct link between AKI and the long-term impairment of oxalate homeostasis in rats.

Conclusions. AKI has long-term negative effects on the quantitative and qualitative characteristics of ODB in fecal microbiota disrupting oxalate homeostasis in rats. Moreover, the results of our study confirmed an increasing trend in total fecal ODA according to the aggravation of renal interstitial fibrosis in rats. Further studies are warranted to gain more insight into the mechanism of oxalate homeostasis impairment in AKI.

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Ethical Considerations. The study was carried out in accordance with the Declaration of Helsinki and was approved by a local Ethics Committee.

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Author Contributions:

Natalia Stepanova: conceived the presented concept, designed the study, analyzed and interpreted the data, and was a major contributor to writing the manuscript;

Ganna Tolstanova: conceived the presented concept, designed the study, and edited the final manuscript;

Iryna Akulenko: experiment and methodology;

Valentyn Nepomnyashchii: histopathological evaluation and data interpretation;

Svitlana Savchenko: laboratory measurement;

Alexander Zholos and Mykola Kolesnyk: final manuscript editing and research management. All authors discussed the results and commented on the manuscript.

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