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Phage therapy of Cronobacter-induced urinary tract infection in mice

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: *Cronobacter* spp. is an opportunistic pathogen causing rare but dangerous cases of meningitis, sepsis and urinary tract infection. Phage therapy overcomes antibiotic resistance and represents an alternative approach to standard antimicrobial treatment. There are no published studies on the use of phages against *Cronobacter* spp. *in vivo*. The aim of our study was to prove the effects of isolated *Cronobacter*-specific phages on renal colonization in a model of urinary tract infection in mice.

Material/Methods: Urinary tract infection was induced by transurethral application of *Cronobacter turicensis* (10^{11} CFU/ml). Simultaneously, isolated *Cronobacter*-specific phages were administered intraperitoneally (10^{11} PFU/ml). After 24 hours, kidneys and bladder were collected and used for cultivation and analysis of gene expression and oxidative stress markers.

Results: Phage therapy reduced the number of *Cronobacter* colonies in the kidney by 70%. Higher levels of malondialdehyde were reduced by phage therapy without affecting the antioxidant status. The expression of pro-inflammatory cytokines tumor necrosis factor-alpha and monocyte chemoattractant protein-1 increased by the infection and was attenuated by phage therapy.

Conclusions: Phage therapy proved effective in the prevention of ascending renal infection in a murine model of urinary tract infection. Long-term effects and safety of the treatment are currently unknown. Further studies should test phage therapy in other *Cronobacter* infection models.

key words: **urinary tract infection • *Cronobacter* spp. • *Enterobacter sakazakii* • phage cocktail • oxidative and carbonyl stress**

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Background

Cronobacter spp. (previously known as *Enterobacter sakazakii*) is a Gram-negative opportunistic pathogen belonging to the Enterobacteriaceae family. It is known to cause serious infections including bacteremia, enterocolitis, meningitis and even sepsis, especially in premature newborns [1]. Recently, a case study reporting cystitis caused by *C. sakazakii* in the elderly has been published [2]. The lethality of *Cronobacter* infections is very high (40–80%) and the incidence is relatively low (8.7 per 100 000 low birth weight neonates) [3,4]; however, it is assumed that the number of infections caused by *Cronobacter* is underreported. Antibiotic therapy is effective if applied soon after infection, but resistance against some therapeutics has already been reported [5]. It can be expected that alternative approaches will be needed soon, since the antimicrobial resistance is rising [6].

Powdered milk formulas are an important source of *Cronobacter* infections [7]. The resistance of *Cronobacter* strains to higher temperature and osmotic stress during desiccation enables their survival in dried food [8]. Other virulence factors include production of proteases, endotoxins and outer membrane proteins [9,10]. In addition, *Cronobacter* strains have adhesive properties and are able to invade human cells [11,12].

The current threat of antibiotic resistance has led to re-evaluation of bacteriophages as antibacterial agents potentially usable for treatment and/or prevention of bacterial infections [13,14]. Advantages of this approach are the specificity of phages bypassing the collateral damage induced by antibiotics on natural microflora, and the virtually single-administration efficacy of lytic phages. Phage therapy of staphylococcal and enterococcal infections has already been tested in clinical trials, with few reported unwanted side effects independent of the route of application [15,16]. Studies testing phage cocktails for the treatment of chronic venous leg ulcers and chronic ear infection are currently in progress [17,18]. Additionally, promising results of animal experiments show that phages might play a role in the biocontrol of pathogens in clinical medicine [19].

It has been demonstrated *in vitro* that contamination of powdered milk formulas with *Cronobacter* can be effectively eliminated with selected *Cronobacter*-specific phages [20,21]. To our knowledge, the potential of phage therapy to control *Cronobacter* infection in animal models of human diseases has not yet been tested; therefore the aim of our study was to prove the effects of phage therapy on *Cronobacter*-induced urinary tract infection (UTI) in a murine model.

Material and Methods

Bacterial strains and phages

The bacterial strain used in this study was isolated from a food matrix (obtained from Food Research Institute, Bratislava) in Luria-Bertani (LB) medium overnight at 37°C. The Strain *C. turicensis* 290708/07 was identified using the biochemical API 20E identification system (Biomerieux, Marcy-l'Etoile, France) and characterized by molecular methods as described in detail by Turcovsky et al. [22]. This strain was used as a representative *Cronobacter* spp. strain for phage propagation, isolation and examination throughout the study. For *in vivo* experiments, bacteria were centrifuged (6000 g/10 min/4°C) after overnight cultivation and resuspended in phosphate buffer saline (PBS). The number of colony-forming units was determined by standard plate counts of serial dilutions on LB agar or MacConkey agar plates.

Phages used in the study were isolated from sewage collected from 2 wastewater treatment plants in Petržalka and Devínska Nová Ves, Bratislava, Slovakia (further in text as P2, D2, respectively). Pure phage cultures were obtained by repeated isolation from single plaques grown on indicator strain in double agar overlay plates. Phages were further purified by ultracentrifugation on the sucrose gradient. A mixture of the 2 phages with the highest lytic activity toward the indicator strain was used in the *in vivo* experiment.

In vitro specificity of phages

The ability of phages to infect different strains was assessed by the spot test. Incubated indicator bacterial lawns were dropped with 10 µl (10^8 PFU/ml) of phage preparation, following incubation at 37°C overnight. On the next day, bacterial lawns were examined for lysis zones. The bacterial strains used in this test contained – 10 *E. coli* strains, 24 *Enterobacter cloacae* strains, 2 *Citrobacter* strains and 7 different *Salmonella enterica* strains.

Animal model of urinary tract infection

Forty-seven C57BL/6NCrl 10-11-week-old female mice were used for the experiment (AnLab Ltd., Prague, Czech Republic). Animals were housed in a room with a 12/12 h light dark cycle and had ad libitum access to water and food pellets. Animals were divided into 4 groups as follows: CRONO (n=13) with *C. turicensis* transurethral application, CRONO-PHAGE (n=14) with *C. turicensis* transurethral application and intraperitoneal phage administration, PHAGE (n=10) with intraperitoneal phage administration and a CTRL group (n=10) as a mock-infected control group. All experiments were approved by the Ethics Committee of the Institute of Molecular Biomedicine, Comenius University in Bratislava, Slovakia.

UTI was modeled according to a recently published protocol [23]. The mice were briefly anaesthetized with isoflurane and instilled via 28G plastic transurethral catheters (outer diameter 0.35 mm, inner diameter 0.28 mm) with a volume of 100 µl containing either 1×10^{11} colony-forming units/ml of *C. turicensis* or sterile LB medium in the experimental group. Simultaneously, 100 µl of either *Cronobacter* – mixture of phages (1×10^{11} plaque-forming units/ml), or sterile PBS were administered intraperitoneally. After 24 h blood, bladder and kidneys were collected under aseptic conditions. Tissues were homogenized in 300 µl of sterile PBS using TissueLyser II (Qiagen, Hilden, Germany), and aliquots were used for cultivation and biochemical analyses.

Biochemical analyses

All chemicals were from Sigma-Aldrich (St Louis, USA), if not otherwise stated. Advanced glycation end-products (AGEs) as markers of carbonyl stress were determined

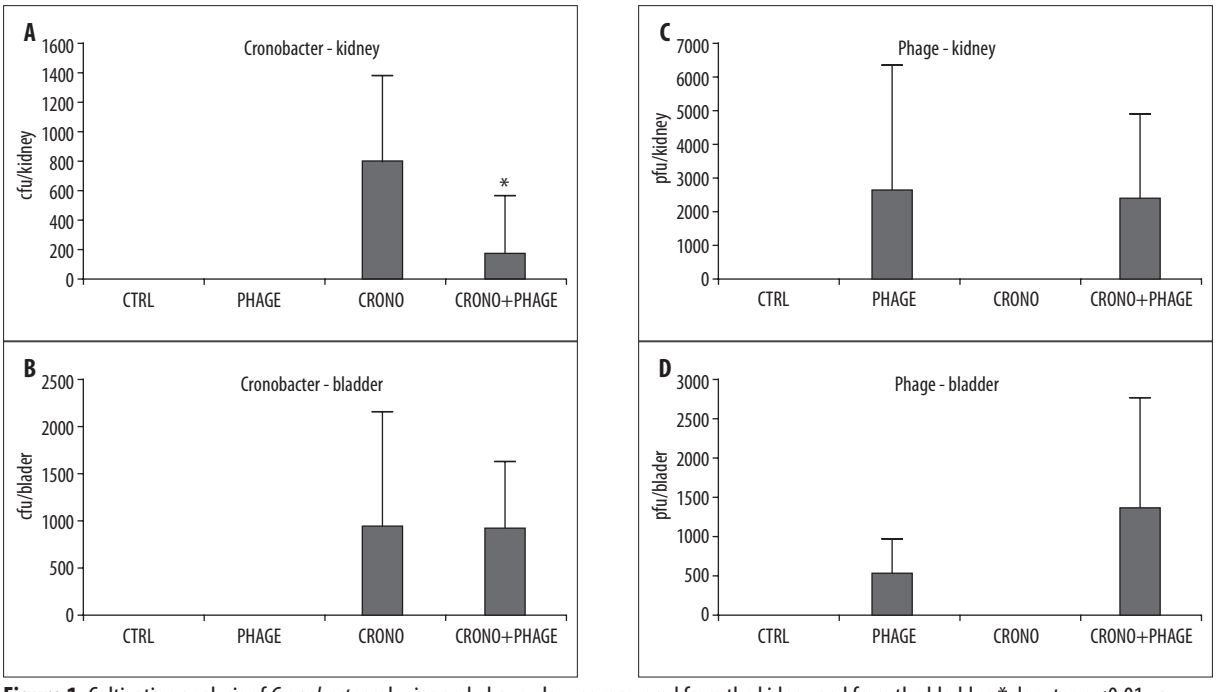

Figure 1. Cultivation analysis of *Cronobacter* colonies and phage plaques recovered from the kidney and from the bladder. * denotes p<0.01 *vs.* CRONO.

spectrofluorometrically, using the specific fluorescence of Schiff base and Amadori products after addition of phosphate buffer saline (PBS) at λ_{ex}=370 nm and λ_{em}=430 nm directly in homogenate tissues [24]. Thiobarbituric acid reacting substances (TBARS) as a marker of oxidative damage of lipids were determined as described by Ohkawa et al. [25]. Briefly, 20 µl of homogenate, 30 µl distilled H_2O, 20 µl of 0.67% thiobarbituric acid (Sigma-Aldrich, Germany) and 20 µl of glacial acetic acid were mixed in 96-well microtitration plate. After 45 minutes of incubation at 94°C (Mastercycle pro S, Eppendorf, Hamburg, Germany), 100 µl of n-butanol (Merck, USA) was added and the absorbance was measured after centrifugation (2000 g/10 min/4°C) in the upper phase at 532 nm. Ferric reducing activity of the tissue (FRAT) as a marker of antioxidant status was measured as described by Erel et al. [26]. Twenty µl of sample was added into 200 µl of freshly prepared FRAT reagent (3 mol/l acetate buffer, pH 3.6; 10 mmol/l of tripyridyl-s-triazine dissolved in 40 mmo/l HCl; 20 mmol/l $FeCl_3$; and $6H_2O$ and H_2O in ratio 1:1:1:9, respectively) warmed to 37°C and measured after 4 min of incubation at 593 nm. Proteins were quantified using the Bradford method [27]. As a standard, bovine serum albumin (Fermentas, Vilnius, Lithuania) was used and 5 µl of tissue homogenates were mixed with 250 µl of Bradford reagent (Fermentas, Vilnius, Lithuania). Absorbance was measured at 595 nm after 5 min. All measurements were done on a Sapphire II instrument (Tecan, Grödig, Austria).

Real-time PCR

Total RNA was isolated from tissues using the TriReagent (MRC, Cincinnati, USA). Concentration and purity of isolated RNA was evaluated spectrophotometrically by Nanodrop (Thermo Fisher Scientific, USA). One-step RT real-time PCR with specific primers was used for the relative quantification of gene expression (QuantiTect SYBR green RT-PCR kit, Qiagen, Hilden, Germany). Genes of interest were tumor necrosis factor-alpha (TNF-α; primer F – gtccctttcactcactggcc; primer R – gagtgcctcttctgccagttc) and monocyte chemoattractant protein-1 (MCP-1; primer F – cctacaactttattaaaactgcatctg; primer R - ctagttcactgtcaactggtcactc). Peptidylprolyl isomerase A (PPIA; primer F – ttcgagctctgagcactgg; primer R – ccagtgccattatggcgt) was used as a housekeeping gene.

Statistical analysis

The results were analyzed using the XLStatistics v. 10.05.30 software. Differences between groups were evaluated with ANOVA and post-hoc Student t-test with Bonferroni correction of multiple comparison bias. P-values less than 0.05 were considered significant. Data are presented as mean ± standard deviation.

Results

Electron microscopy of used bacteriophages P2 and D2 showed that both are members of the *Podoviridae* family, order *Caudovirales,* with 180±20 nm head diameter and 60±10 nm long non-contractile tail. Host specificity tests were performed on 41 different strains, including *Salmonella, E. coli, Citrobacter* and *Enterobacter cloacae.* Neither of these phages was able to infect *Citrobacter, Salmonella or E. coli* strains. Phages showed different ability to lyse *Enterobacter cloacae* strains (P2 – 25%, D2 – 38%).

Cultivation analysis revealed that bacterial colonies were found only in bladders and kidneys of mice that were injected with *Cronobacter* cells. Mean bacterial count in the kidney was 734 cfu and phage therapy decreased the count by 70% (Z=3.06; p=0.002, Figure 1A). In the bladder the mean bacterial load was cca 900 cfu in both CRONO and CRONO + PHAGE groups (Z=–0.55; p=0.58; Figure 1B). No significant

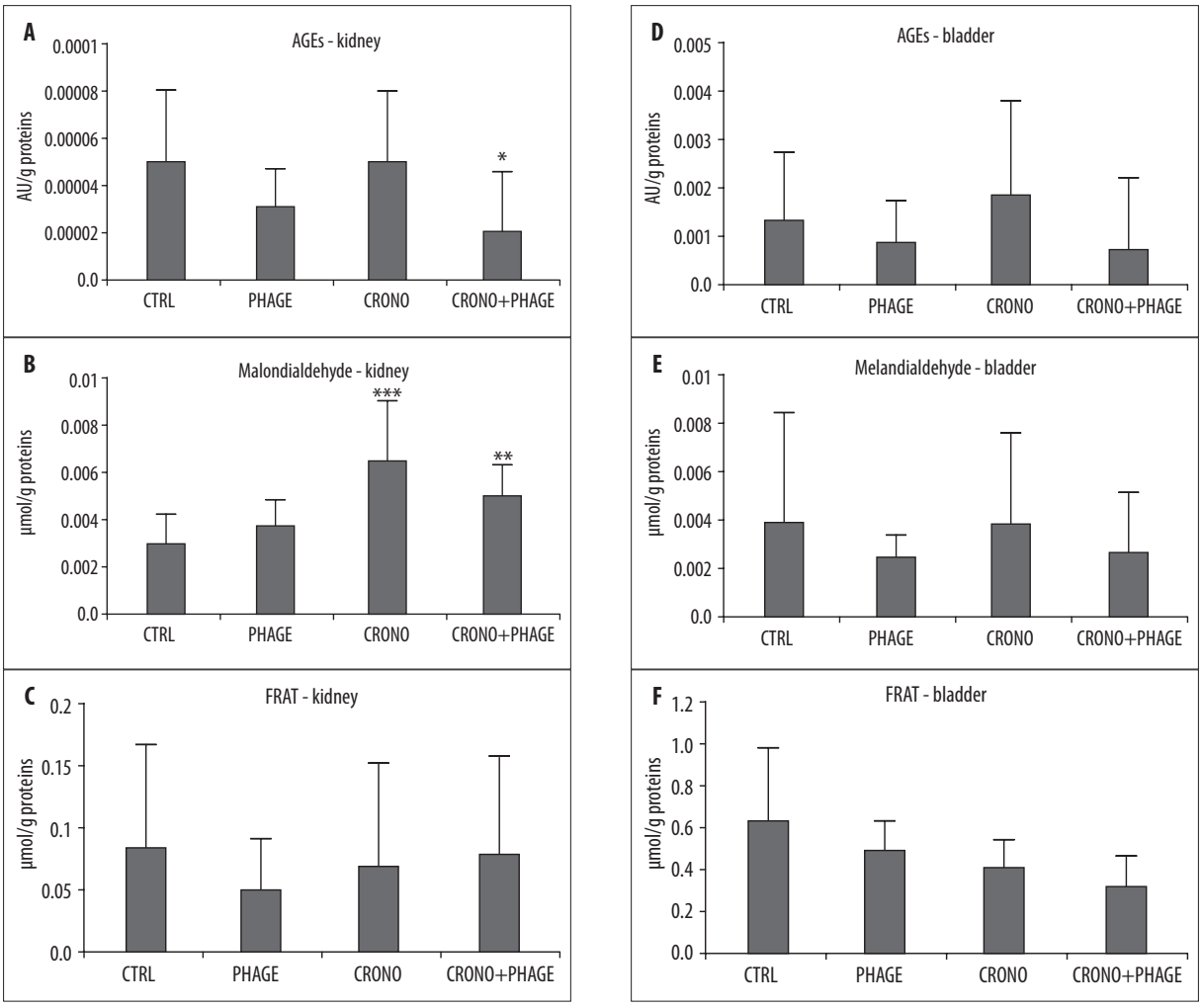

Figure 2. Oxidative stress markers in the kidney and in the bladder. *** denotes p<0.001 *vs.* CTRL; * denotes p<0.05 *vs.* CRONO; ** denotes p<0.01 *vs.* CRONO.

differences were found between the PHAGE and CRONO + PHAGE groups in the number of phage plaques in the kidney (Z=0.32; p=0.75; Figure 1C), but were higher in the CRONO + PHAGE group, although not significant due to high interindividual variability (Z=–1.53; p=0.13; Figure 1D).

AGEs in renal cortex as a marker of carbonyl stress were not altered by infection with *Cronobacter*, but were significantly reduced by phage therapy in comparison to the infected mice (by 57%; t=2.46; p=0.02; Figure 2A). Malondialdehyde as a marker of lipoperoxidation was doubled in the kidneys of infected mice (t=4.15; p=0.0009; Figure 2B) and lowered with phage therapy by 39% (t=2.94; p=0.008; Figure 2B). Groups did not differ in FRAT as a marker of antioxidant status in the renal tissue (Figure 2C). No significant differences were found in any of the analyzed markers of oxidative stress in the bladder (Figure 2D–F).

Analysis of expression of TNF alpha and MCP-1 revealed that both cytokines were upregulated in the bladder of infected mice (TNF alpha by 72%; t=3.02; p=0.007; MCP-1 by 52%; t=3.03; p=0.007). These differences were reduced by phage therapy (TNF alpha by 35%; t=3.58; p=0.002; Figure 3C; MCP-1 by 22%; t=2.53; p=0.02; Figure 3D). In the renal cortex only the infection-induced increase in expression of MCP-1 was significant (by 48%; t=2.57; p=0.02; Figure 3B). The decrease due to phage therapy was significant for TNF alpha (by 36%; t=2.78; p=0.02; Figure 3A).

Discussion

Although seldom life-threatening, chronic UTI is associated with high morbidity and thus represents a frequent problem in primary care [28]. It even represents a common cause of sepsis in critically ill or otherwise immuno-compromised patients and may be a cause of nonspecific complaints with negative initial urinalysis in the elderly [29]. In most cases, appropriate choice and dosing of antibiotics or chemotherapeutics is sufficient for successful treatment; however, with increasing incidence of UTI caused by resistant strains, the need for alternative options becomes urgent [30].

Bacteriophages as viruses that attack bacteria are such an alternative. Phages with a lytic cycle can be seen as natural antimicrobial agents. Host specificity of phages is high and can be narrowed by genetic engineering or by *in vitro* evolution and selection [31]. The safety profile seems to be far better in comparison to antibiotics, at least according to

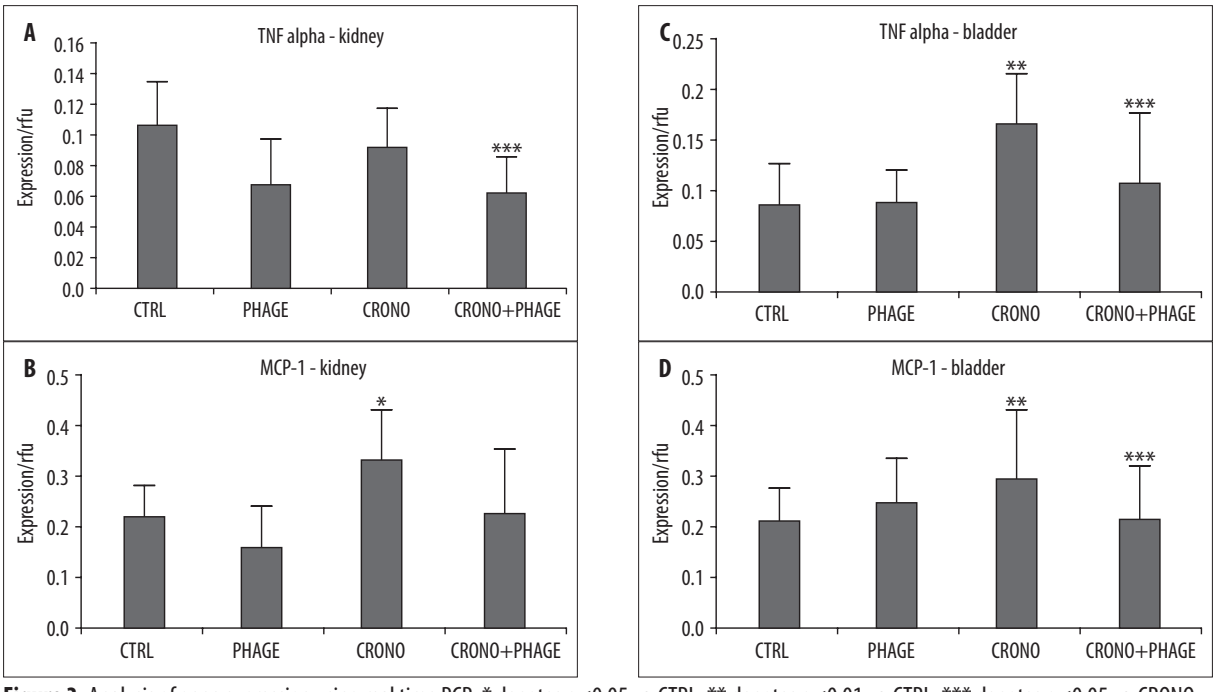

Figure 3. Analysis of gene expression using real time PCR. * denotes $p<0.05$ *vs.* CTRL; ** denotes $p<0.01$ *vs.* CTRL; *** denotes $p<0.05$ *vs.* CRONO.

already published data. Phage particles do not invade or actively affect eukaryotic cells, but may induce a humoral immune response [18]. Long-term studies dealing with safety of phage administration are, nevertheless, needed [19].

UTI caused by *Cronobacter* spp. is rather rare [2]. However, UTI in mice is a suitable model for testing the ability of phage therapy to conquer an infection *in vivo*, mainly due to physiologically sterile environment and a reproducible read-out parameter in the form of renal colonization. Ascending nephritis was partially prevented in our experiment, indicating that the applied phages could be usable in other infection models. This assumption has to be verified in separate studies, as the conditions in the urinary tract differ from those in other organs or tissues. The observed 70% reduction of *Cronobacter* colonies in the kidneys 24 hours after a single phage dose is consistent with other similar results observing phage efficacy in the treatment of wound infections [32]. The ability of phages to survive in the urinary tract 24 hours after administration has already been shown [33]. There was no difference in *Cronobacter* colonization of the bladder between treated and infected groups. This might indicate that the kinetics of phages after intraperitoneal administration could be improved by choosing another application route. On the other hand, similar to other studies using the transurethral UTI model, the variance, especially in the cultivation analysis, is high. In addition to simple reproduction of the results, a similar confirmatory experiment is planned on larger experimental animals, including rats, dogs and other vertebrates [23].

According to our knowledge this is the first study showing that phage therapy decreases oxidative stress in urinary tract infection. It has been shown that phage therapy can attenuate the effects of oxidative burst caused by activated phagocytes [34]. Whether the observed reduction in oxidative stress in our experiment is direct or due to reduced inflammation cannot be determined based on the available data. The clinical significance of this antioxidative effect is questionable, but might gain importance in chronic infections where oxidative damage of tissues plays an important role in the pathogenesis.

Conclusions

Data from the presented study show that the phage therapy was effective in the prevention of renal infection in a model of *Cronobacter*-induced urinary tract infection in mice. Inflammation and oxidative stress were reduced in the kidney and partially reduced in the bladder. Future studies should focus on long-term effects and safety, as well as on the potential use of phage therapy of *Cronobacter* infections in models of enterocolitis, meningitis or sepsis.

Disclosure

No conflict of interest to declare.

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