Eur J Clin Microbiol Infect Dis (2014) 33:2149–2156
DOI 10.1007/s10096-014-2176-x

ARTICLE

Upregulation of IFN-γ and IL-12 is associated with a milder form of hantavirus hemorrhagic fever with renal syndrome

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Received: 30 April 2014 / Accepted: 29 May 2014 / Published online: 19 June 2014

Abstract Hantavirus hemorrhagic fever with renal syndrome (HFRS) is a zoonotic disease characterized by acute onset, fever, malaise, and back pain. As the disease progresses, hemorrhagic disturbances and kidney dysfunctions predominate. The examination of tissue collected postmortem supports the premise that virus replication is not responsible for this pathology; therefore, it is widely believed that virus-induced immune responses lead to the clinical manifestations associated with HFRS. The overproduction of inflammatory cytokines is commonly reported in subjects with HFRS and has given rise to the hypothesis that a so-called "cytokine storm" may play a pivotal role in the pathogenesis of this disease. Currently, supportive care remains the only effective treatment for HFRS. Our data show that serum levels of interferon (IFN)-γ, interleukin (IL)-10, CCL2, and IL-12 are upregulated in HFRS cases when compared to healthy controls and the level of upregulation is dependent on the phase and severity of the disease. Furthermore, we observed an association between the mild form of the disease and elevated serum levels of IFN-γ and IL-12. Collectively, these observations suggest that the administration of exogenous IFN-γ and IL-12 may provide antiviral benefits for the treatment of HFRS and, thus, warrants further investigations.

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Introduction

Hantavirus hemorrhagic fever with renal syndrome (HFRS) is a febrile disease characterized by bleeding and renal pathology [1, 2]. The onset of the disease involves flu-like symptoms, including fever, headache, and back pain [2], and laboratory results typically reveal thrombocytopenia, lymphocytosis with decreased CD4:CD8 ratio, increased B lymphocyte counts, and proteinuria [2–7]. Additionally, changes in blood chemistry are usually observed early in the course of the disease; for instance, an increased hematocrit and a decrease in serum protein levels are commonly reported during the first week of onset [8–10]. As the disease progresses, hemorrhages appear, which vary from small petechiae to severe internal bleeding [11, 12]. Severe cases often develop disseminated intravascular coagulation (DIC) syndrome, which is considered the primary cause of death in those with HFRS [13–15]. Kidney pathology is described in all cases, and progresses through several stages of kidney dysfunction, including oliguric, polyuric, and convalescence phases [16, 17]. Nevertheless, kidney failure is not common and is usually associated with the most severe cases [17]. Although various degrees of bleeding and kidney dysfunction are commonly associated with HFRS, the pathogenesis of the disease remains poorly defined.

Puumala virus belongs to the species of hantavirus typically associated with HFRS in the Tatarstan region of Russia [18]. Although endothelial cells are believed to be the primary targets of Puumala virus infection, cytopathicity [19, 20] and tissue damage [20] are not observed in specimens collected

postmortem. Several lines of evidence suggest that unknown immune mechanisms may play a key role in the pathogenesis of HFRS. For example, early activation of cytotoxic T lymphocytes (CTLs) has been observed in HFRS cases [7, 21, 22]. Also, immune complexes are often found in the serum before hantavirus-specific antibodies can be detected [23, 24]. Lastly, high levels of proinflammatory cytokines have been detected in the serum of those with HFRS and cytokine-producing cells have been observed in tissue biopsies collected from these subjects. For these reasons, high levels of proinflammatory cytokines are considered a hallmark of HFRS [24–26].

Increased serum levels of tumor necrosis factor (TNF)-α have been reported by numerous investigators [27–29], suggesting a central role for this cytokine in the pathogenesis of HFRS. Consistent with this observation, in vitro TNF-α treatment of endothelial cell monolayers results in an increased permeability without visible cytopathic effects [30, 31]. Previous studies have shown that TNF-α can lead to postcapillary venular leakage in vivo and increased vascular permeability [32–34]. Therefore, it has been proposed that the increased vascular permeability and bleeding associated with hantavirus infection might result from the actions of proinflammatory cytokines. Indeed, a large body of data supports the hypothesis that bleeding and kidney dysfunction are a result of the of the "cytokine storm" often associated with HFRS.

In this study, we sought to determine if the serum cytokine profiles of HFRS cases differ between early and convalescent phases of the disease. We have found that levels of interferon (IFN)-γ, interleukin (IL)-10, CCL2, and IL-12 (p70) were increased in HFRS cases when compared to controls and that differences in cytokine levels were dependent on the phase of infection and severity of the disease. The mild form of HFRS was characterized by a significant increase of IFN-γ. Also, a correlation was demonstrated between the mild form of the disease and serum IFN-γ and IL-12 (p70). Serum CCL2 was also upregulated in HFRS cases, suggesting a potential role for this cytokine in the pathogenesis of this disease.

Materials and methods

Subjects

Twenty-nine subjects (24 male and five female) who were admitted to the Agafonov Republican Clinical Hospital for Infectious Disease, Republic of Tatarstan were enrolled into this study. The diagnosis of HFRS was established based on clinical presentation and was serologically confirmed by the detection of anti-HFRS antibodies. Serum samples from 12 healthy individuals were collected and served as controls. The Institutional Review Board of the Kazan Federal University approved this study and informed consent was obtained from each study subject according to the guidelines approved under this protocol (article 20, Federal Law "Protection of Health Right of Citizens of Russian Federation" N323-FZ, 11.21.2011). The clinical characteristics of HFRS cases are summarized in Table 1.

The Hantagnost diagnostic ELISA kit (Institute Poliomyelitis and Encephalitis, Russia) was used to detect hantavirus-specific antibody titers. This kit allows for the detection of all species of hantavirus common to the Volga region of Russia. Two serum samples were collected from each case for the purpose of diagnosis; the first blood draw was collected at the time of admission and the second was collected 7–10 days later. Subjects were considered positive when the serum anti-hantavirus antibody titers of the second draw were increased four-fold over the first. Separate blood draws were made for cytokine analysis. All 29 subjects provided at least one serum specimen for the purpose of cytokine analysis and 14 of these subjects provided two serum specimens for cytokine analysis. Because two separate blood draws for cytokine analysis were required for the longitudinal analysis, only clinical data are provided for these 14 subjects. Serum specimens were available from 27 cases at the early stage of the disease and 15 cases at the late stage of the disease.

Specimens

Subjects' blood was collected for cytokine analysis into serum-separator tubes and aliquots of 100 μL were made and stored at −80 ° C until analyzed. Serum from cases was collected at a single time point during the disease or at two time points, one at the time of admission and one before discharge from the hospital. A single time point was collected for each control subject.

Cytokine analysis

Levels of serum IFN-γ, IL-1β, IL-2, CXCL8 (IL-8), IL-10, IL-12 (p70), and CCL2 (MCP-1) were analyzed on a Luminex platform (Luminex, Austin, TX), using the respective Singleplex cytokine kit (Bio-Rad, Hercules, CA), according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was conducted using Minitab software (Minitab, State College, PA); differences between the medians of compared groups were performed using the Mann–Whitney test for non-parametric data and were considered significant at $p \leq 0.05$. R-square values for regression analysis between two respective parameters are reported to demonstration goodness of fit.

Table 1 Clinical characteristics of hantavirus hemorrhagic fever with renal syndrome (HFRS) cases

Variables	Value	*p*-Value
Antibody titer (1st)	895±105	
Antibody titer (2nd)	2,041.5±25	
Hospitalization (days)	15.7±2.4	
Febrile phase (days)	4.5±1.2	
Oliguric phase (days)	6.7±1.9	
Serum urea (mg/dL)	60.2±25.1	>0.05
Serum creatinine (μmol/dL)	229.4±19.4	<0.006
Platelet count (1st)	159.4±32.1	>0.05
Platelet count (2nd)	449.9±40	>0.05
Bleeding	6 out of 14	
Kidney insufficiency/edema*	8 out of 14	
Sex (M/F)	12/2	

*Ultrasound diagnosis

Results

Clinical presentation

Twenty-nine HFRS cases (24 male and five female) were recruited in this study (Table 1). The average hospitalization period was 15 days and the average duration of febrile and oliguric periods were 4.5±1.2 and 6.7±1.9, respectively. HFRS diagnosis was based on clinical presentation, epidemiological data, and serological confirmation. Urinalysis revealed proteinuria and hematuria in 3 and 4 out of the 29 total cases, respectively. Ultrasound analysis determined swelling of the kidneys and signs of pyelonephritis. Seven cases developed complications, including disseminated intravascular coagulation and acute renal failure. Increased levels of serum creatinine and urea were detected in 16 and 17 cases, respectively. Platelet counts were decreased at the early stage of the disease (159.4±32.1), while it was restored to that of healthy controls (449±40) by the end of their hospitalization.

Serum cytokine levels

In this study, we evaluated the following cytokines and chemokines based on the phase of the disease: IFN-γ, IL-1β, IL-2, CXCL8, IL-10, IL12 (p70), and CCL2. Two serum specimens were collected from 14 cases, while a single specimen was collected from 15 cases. The first specimen was collected at the time of admission and the second was collected during the course of the disease or before the subject was discharged from the hospital. Significantly decreased levels of IL-2 and IL-12 (p70) characterized the febrile phase of the disease, while levels of IL-10 and CCL2 were increased compared to that of healthy controls (Table 2). During the oliguric phase, levels of IL-2 and IL-12 (p70) remained significantly decreased, while serum IL-10 and CCL2 were upregulated to a similar level to that found during the febrile phase. In addition, levels of IL-1β were found to be downregulated, while levels of the chemokine CXCL8 were marginally increased compared to that in the serum of healthy controls. There were no changes in levels of IL-2, IL-12 (p70), IL-10, and CCL2 during the febrile phase and levels remained similar to that of the polyuric phase of the disease. During the convalescent phase, levels of serum IFN-γ as well as IL-10 and CCL2 were significantly increased, while serum levels of IL-1β and IL-12 (p70) remained significantly decreased compared to healthy controls. These data suggest that, during HFRS, significant changes in the serum levels of several cytokines and chemokines are involved in the regulation of inflammation and the activation of immune responses.

Two groups of cases are identified based on levels of serum IFN-γ and IL-12 (p70)

Further analysis of serum cytokine levels, from specimens collected during the course of the disease (second collection), revealed that two groups of cases could be stratified based on their level of IFN-γ (Table 3). The first group (Group 1) was characterized by increased levels of IFN-γ above that of healthy controls (123.5±25 vs. 20±7 ng/mL, respectively, $p=0.0034$), while the second group (Group 2) displayed IFN-γ levels (12.3±5.1) comparable to that of the control group. Elevated serum IFN-γ is commonly associated with the activation of CTLs; therefore, we sought to determine if Group 1 and Group 2 differed in levels of IL-12 (p70), which has been shown to be upregulated during CTL-type immune responses [35]. Statistical analysis of medians did not reach significance for IL-12 (p70) between the two groups ($p=0.053$), so we next sought to determine if the severity of the disease differed between the two groups of cases. The duration of febrile and oliguric phases, as well as the hospitalization period, were used to assess the severity of the disease. Group 1 and Group 2 differed significantly in all selected parameters when analyzed. Febrile and oliguric phases of the disease were significantly shorter in Group 1 as compared to Group 2 ($p<0.02$ and $p<0.001$, respectively). Also, the duration of hospitalization was significantly shorter in Group 1 compared to Group 2 ($p=0.019$). Furthermore, the second group had a higher frequency of bleeding (80 % vs. 0 % in Group 1) and kidney insufficiency (80 % vs. 44.4 % in Group 1).

These data suggest that cases with levels of serum IFN-γ above that of the control group tend to have less severe disease. Because the duration of the oliguric phase is the principal clinical parameter indicating kidney damage, and also serves as an indicator of disease severity, we hypothesized that a correlation may exist between serum IFN-γ and the oliguric phase. Indeed, regression analysis demonstrated a

Table 2 Serum cytokine concentration in different phases of HFRS

	Healthy control	HFRS (phase)			
		Febrile	Oliguric	Polyuric	Convalescent
IFN-γ	20±7	12.6±1.8	9.7±5.2	1.9±0.5	50.9±29
		<0.02	<0.03	$<6.6 \times 10^{-8}$	
IL-1β	25.3±5.0	13.5±4.0	1.4±0.8	16.2±4.1	6.8±3.1
			<0.001		<0.01
IL-2	12.2±2.1	2.4±1.5	3.2±0.6	3.5±4.2	24.6±9.8
		<0.002	$<3.9 \times 10^{-5}$	<0.02	
CXCL8	218.0±58	376.5±120	493.9±156	191.4±45	253.4±36
			<0.05		
IL-10	26.4±4.1	70.8±4.2	132.9±10.2	53.4±25	42.7±4.6
		<0.0006	<0.002		<0.03
IL-12 (p70)	352.2±28	5.8±2.5	5.6±2.3	2.3±10	32±4.7
		$<3.8 \times 10^{-7}$	$<9.8 \times 10^{-13}$	$<2.1 \times 10^{-6}$	$<1.8 \times 10^{-10}$
CCL2	994.1±35	9451.4±178	7533.1±215	5554.5±124	5228.1±115
		<0.005	$<4.5 \times 10^{-5}$	$<1.0 \times 10^{-6}$	$<4.1 \times 10^{-5}$

significant correlation between IFN-γ and duration of the oliguric phase (R-square=78.1 %), thus supporting our hypothesis. We also sought to establish if a correlation existed between the oliguric phase and serum IL-12 (p70). Although the correlation between the oliguric phase in IL-12 (p70) was weak (R-square=38 %), if both IFN-γ and IL-12 (p70) were considered together as two independent variables [oliguric phase=IL-12 (p70)+ IFN-γ], an even greater correlation was observed (R-square=84.7 %), beyond that of IFN-γ alone. These data suggest that both IFN-γ and IL-12 (p70) influence disease severity but likely work though different mechanisms.

Differences in the clinical presentation of cases in Group 1 and Group 2

Although the average levels of serum creatinine and urea were higher in Group 2 as compared to Group 1, these differences were not statistically significant. Additionally, the platelet

Table 3 Clinical presentation, laboratory parameters, and cytokines of HFRS cases in Group 1 and Group 2

Variables	Group 1	Group 2	Healthy control
IFN-γ	123.5±25	12.3±5.1	20±7
p-Value	<0.0034	<0.001	
	<0.02*		
IL-12 (p70)	131.3±60.1	29.8±40.2	352.2±28
Antibody titer (1st)	349±40	80±35	
Antibody titer (2nd)	985.6±39.1	768.2±35.2	
Hospitalization (days)	14.3±1.1	18.2±1.2	
p-Value	<0.019*		
Febrile phase (days)	3.9±1.2	5.6±0.5	
Oliguric phase (day)	5.4±1.8	9.0±1.0	
Serum urea	13.5±4.2	116.2±79	66.3±20.3
p-Value	<0.004		
Serum creatinine	187.0±20.5	293±98	5.2±2.1
p-Value	<0.05	<0.001	
Platelet count (1st)	205±67	77.2±79	283±45
p-Value	<0.5	<0.005	
Platelet counts (2nd)	474.1±26	406.4±58	283±45
p-Value	<0.02		
Bleeding	0/9 (0 %)	4/5 (80 %)	
Kidney insufficiency/edema	4/9 (44.4 %)	4/5 (80 %)	

*p-Value between Group 1 and Group 2

count decrease was more pronounced in Group 2 than in Group 1 (77±vs. 205, respectively); however, again, the differences were not statistically significant. There was no difference between Group 1 and Group 2 in the number of platelets during the convalescent phase of the disease (496 vs. 406, respectively). Cases in Group 2 had a higher frequency of erythrocytes in their urine (4 out of 6; 66 % vs. 0 out of 9; 0 %), suggesting that those in Group 2 were more likely to have developed clinical signs of kidney damage.

Discussion

HFRS is endemic in Russia, with 10,000–12,000 clinical cases registered annually in the European part of Russia alone [36]. The majority of HFRS cases have been registered in the Volga Federal District, especially Tatarstan, Udmurtia, Samara, Orenburg, and Bashkortostan [18]. It has been shown that Puumala virus infection is main cause of the disease in the Western part of Russia, while sporadic cases of Dobrava virus infection are also registered [18, 36]. Puumala virus-associated HFRS is commonly referred to as nephropathia epidemica (NE) and clinically presents with milder symptoms when compared to hantaviruses in other parts of the world [37].

In the present study, 29 HFRS cases were recruited; their diagnosis was based on clinical presentation, epidemiological data, and serological confirmation. Increased levels of serum blood urea nitrogen (BUN) and creatinine characterized HFRS in this study. Platelet counts were decreased early during the course of the disease and returned to normal levels at the convalescence stage. As is typical with HFRS, subjects in this study displayed varied levels of bleeding; four cases were observed to have petechiae and red blood cells in the urine, while three cases displayed DIC syndrome.

It is widely believed that HFRS pathogenesis is driven by a "cytokine storm", as increased levels of serum proinflammatory cytokines are commonly observed [25–27]. For instance, elevated levels of serum IL-10 and sIL-2R have been reported in subjects with HFRS [6, 29, 38–40], and TNF-α, IL-6, and CXCL8 have been reported in the serum of cases during early and late phases of their disease [10, 25–27, 41]. In the present study, we found increased levels of serum CXCL8 and IL-10 in HFRS cases when compared to healthy controls. Levels of IL-2 were decreased early during infection, while they exceeded that of controls at the convalescent phase. Accordingly, our results support the previous observations of other researchers and expand upon these results.

The promotion of CTL responses in individuals with HFRS is well documented and numerous studies have shown the presence of hantavirus-specific CTLs in the blood of convalescent cases [7, 21, 22]. Additionally, $CD8^+$ T cell infiltration has been shown in the kidneys of HFRS cases [42, 43]. These data suggest that CTLs may play a role in the pathogenesis of hantavirus-associated HFRS. The activation of CTLs is tightly controlled and involves IL-12 and IFN-γ. For example, transitory stimulation of $CD8^+$ memory T cells with IL-12 leads to improved antiviral activity [44, 45]. Additionally, IL-12 activates natural killer (NK) cells and promotes specific allogeneic CTL reactions [46, 47]. It has been shown that IL-12 stimulates the production of IFN-γ and TNF-α by NK cells and CTLs [48–51]. With these data in mind, we focused our analysis on IFN-γ and IL-12 expression in HFRS cases. We have found two distinct groups of HFRS cases based on the serum levels of IFN-γ. Those with high levels of IFN-γ had significantly shorter hospitalization periods and duration of febrile and oliguric phases.

Of the different phases associated with HFRS, the oliguric phase is the most critical, as it reflects impairment of kidney function. The rate of kidney filtration markedly declines during the oliguric phase and clinically presents with decreased urine output [52, 53]. Proteinuria and increased serum levels of creatinine and urea also characterize the oliguric phase [52, 53]. Additionally, a decline in the urine output is one of the criteria used to determine the severity of the disease, where the severe form presents with lower urine output [10]. Furthermore, the oliguric phase is considered to be significant because of the high likelihood of developing life-threatening complications [54]. Our data reveal a correlation between high levels of serum IFN-γ and a shorter duration of the oliguric phase. Interestingly, there was only a weak correlation between the duration of the oliguric phase and serum IL-12 (70) levels; however, the correlation was substantially higher between the duration of the oliguric phase and serum levels of IL-12 and IFN-γ when analyzed together. These data suggest that both IFN-γ and IL-12 contribute to the pathogenesis of HFRS. Specifically, IFN-γ and IL-12 (p70) likely reduce kidney damage and facilitate kidney filtration. Additionally, it appears that those with high serum IFN-γ display less prominent signs of kidney damage, including the presence of erythrocytes in their urine, and increased serum levels of creatinine and urea. Taken together, these data suggest that cases with high levels of serum IL-12 (p70) and IFN-γ tend to develop a milder form of the disease. Conversely, cases with low levels of serum IL-12 (p70) and IFN-γ develop a more severe form, characterized by a longer hospitalization and duration of the oliguric phase.

When IL-12 (p70) and IFN-γ were evaluated together, we observed a higher correlation between these cytokines and the duration of the oliguric phase as compared to their analysis individually. This finding suggests that IFN-γ and IL-12 (p70) originate from different sources. IFN-γ is released by a variety of immune cells, including NK cells, natural killer T (NKT) cells, Th1 cells, and CTLs [35]. Although coming from different sources, IFN-γ and IL-12 have synergistic effects on

promoting Th1-type immune responses. For example, IL-12 enhances T cell commitment to IFN-γ production, while IFN-γ promotes a powerful feedback to further stimulate IL-12 production by phagocytic cells [55, 56]. We observed higher levels of serum IFN-γ in cases with a milder form of the disease; therefore, it appears that a type 1 immune response may be associated with less severe disease progression. Indeed, the activation of type 1 immune responses to hantavirus infection has been described by several researchers [7, 21, 22]. Circulating hantavirus-specific CTLs are found in HFRS and hantavirus pulmonary syndrome (HPS) convalescent donors [9, 57–59]. Nevertheless, the role of these cells in the pathogenesis of HFRS remains largely unknown. Our data suggests that the activation of type 1 immunity may play a protective role, preventing tissue damage. Although our data potentially identify a protective role for IL-12 (p70) and IFN-γ, larger studies will likely be necessary in order to completely elucidate the role of type 1 immune response in hantavirus pathogenesis.

IL-10 is a potent inhibitor of IFN-γ production by lymphocytes, helping to maintain T helper cell polarization, promoting a Th2 phenotype [60, 61]. We have found that levels of serum IL-10 were significantly higher in HFRS cases during the febrile, oliguric, and convalescent phases, consistent with the polarization of T helper cells towards a Th2 phenotype. However, significant upregulation of IFN-γ was detected during the oliguric and polyuric phases of the disease. The activation of IFN-γ indicates T helper cell differentiation towards Th1-type lymphocytes; therefore, it appears that Th1- and Th2-type immunity is activated during HFRS. Interestingly, the analysis of IL-10 levels revealed no differences in serum cytokine levels in Group 1 and Group 2, while IFN-γ levels were significantly higher in Group 1 as compared to Group 2. These data suggest that Th2-type immune response is equally activated in both groups, although Group 1 may have more vigorous activation of Th1-type immune response as compared to Group 2. Consequently, we believe that both Th1- and Th2-type immune response are necessary for successful recovery, yet the activation of Th1-type immune response appears to be a pivotal marker defining the severity of the disease.

We also observed significantly increased levels of the chemokine CCL2 in the serum of HFRS cases as compared to that of controls. Although CCL2 levels were increased in both Group 1 and Group 2, the level was slightly lower in Group 1. CCL2 is a chemoattractant, promoting the migration of mononuclear leukocytes, such as monocytes, memory T cells, and dendritic cells, to the site of infection [62]. Studies of the kidney biopsies collected postmortem have revealed lymphocyte, monocyte, and macrophage infiltration of kidney tissue in HFRS cases [20]. Therefore, it could be suggested that CCL2 plays a role in the accumulation of lymphocytes in kidney tissue. In addition, as a chemoattractant, CCL2 plays a significant role in promoting Th2-type immune response. For instance, it has been shown that CCL2 selectively inhibits IL-12 production in dendritic cells and inflammatory macrophages [62–64]. Levels of serum CCL2 differed slightly between Group 1 and Group 2, where cases with a milder form of the disease had lower levels of CCL2, while those with a more severe form of HFRS had higher levels. CCL2 is one of the many chemokines responsible for leukocyte migration to the site of infection. Therefore, slight differences in the level of CCL2 could prove to be significant in the kidneys of HFRS cases with respect to the overall chemoattractive environment. Nevertheless, more studies will be required so as to fully decipher the role of CCL2 in the pathogenesis of HFRS.

In conclusion, levels of CCL2 were observed to be significantly elevated in the serum of HFRS cases. CCL2 may potentially play role in HFRS pathogenesis, since this chemokine is a strong chemoattractant for mononuclear leukocytes. Also, an increase in the serum levels of IFN-γ, IL-10, and IL-12 (p70) was observed in HFRS cases when compared to controls. Differences in cytokine levels were dependent on the phase of infection and severity of the disease. Significant increases in IFN-γ were found in the serum of those cases with a milder form of HFRS. Additionally, a correlation was demonstrated between the mild form of the disease and serum levels of IFN-γ and IL-12 (p70). Although future studies will be needed in order to determine if cytokine activation during HFRS is a reaction to virus replication or the outcome of immune system reactivity to infection, these observations suggest that the administration of exogenous IFN-γ and IL-12 may provide antiviral benefits for the treatment of HFRS and, thus, warrants further investigations.

Acknowledgments This research was supported by the subsidy of the Russian Government to support the Program of Competitive Growth of Kazan Federal University. Some of the experiments were conducted with the support of the Federal Center of Collective Use and Pharmaceutical Research and Education Center, Kazan (Volga Region) Federal University, Kazan, Russia.

Conflict of interest The authors declare no conflicts of interest.

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