Tularemia Progression and it Modulation Including Mortality Remission and Enhancing of Immune System Response Using Asoxime (HI-6)

Miroslav Pohanka[1]*

Oto Pavlis[1]

Kamil Kuca[1]

Jiri Pikula[2]

1 Faculty of Military Health Sciences, University of Defence, Trebesska 1575, 50001 Hradec Kralove, Czech Republic

2 Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Sciences Brno, Palackeho 1/3, 612 42 Brno, Czech Republic

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ABSTRACT

Objective:

Francisella tularensis is an intracellular pathogen causing tularemia disease. Immune system action against tularemia is limited due to lipopolysaccharide covering bacterial cell. Cholinergic anti-inflammatory pathway is a link between parasympathetic nervous system and macrophage assisted immunity. Asoxime (also known as HI-6) is a compound implicated in regulation of acetylcholinesterase as well as acetylcholine receptors. We hypothesize suitability of asoxime to modulate tularemia progression.

Procedure and experiment design:

Laboratory mice BALB/c were infected with F. tularensis LVS strain and challenged by application of 209 μg/kg to 209 mg/kg of HI-6 in the experiment beginning and then the next day. Mice were sacrificed after five days. Plasma, spleen and liver were sampled. In the separate experiment, tularemia caused mortality was assessed with and without of asoxime application.

Results and Conclusions:

Regarding to oxidative damage of liver and spleen, asoxime altered lipid peroxidation in liver and significantly reduced oxidative damage in spleens. We also proved significant increase of plasmatic antibodies level, decrease of IL6 and steady level of IFNγ. Mice treated with asoxime had reduced mortality when compared to the infected and untreated ones. The best protective index was calculated 2.6 for asoxime doses 2.09 and 20.9 mg/kg. Asoxime can be considered as a compound reducing detrimental impact of tularemia. Effect of asoxime on cholinergic anti-inflammatory pathway and overall practical effect is discussed.

INTRODUCTION

Francisealla tularensis is a gram negative bacterium and causative agent of tularemia. The disease manifestation is related to modes of transmission. The ulceroglandular form typically arises after the host is bitten by a tick harbouring the agent or in humans handling infected animals. [1,2] However, tularemia can also elicit oropharyngeal, oculoglandular, septic and pneumonic manifestations. [3-5] Several symptoms accompany the onset of infection. However, after approximately three days patients develop unspecific flu-like symptoms in the first phase. [6] Lymph node enlargement is typical for tularemia; on the other hand, high temperature and fastness of infection progression is quite diverse for individual case reports[7].

Vaccine strains Moscow, 15 and 155 derived from F. tularensis holarctica were prepared and tested in the former Soviet Union. Live vaccine strain (LVS) was developed from strains 15 and 155 in 1950´s.[8] At present, there is no licensed vaccine for tularemia in the USA or European Union. The efficacy of vaccination with killed F. tularensis cells is poor since the lipopolysaccharide (LPS) antigen does not trigger an extensive inflammatory response, stimulate cells of the immune system or bind to toll like receptor - TLR. [9,10] On the other hand, Lavine at al.[11] claimed protective antibody-mediated immunity arising after vaccination with heat-killed LVS. Therapy for tularemia is available because streptomycin, doxycycline, gentamicin, tetracyclines and quinolones are effective antibiotics used in humans. [12-14]

Figure 1

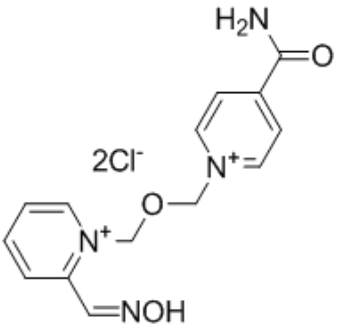
Effects of asoxime on tularemia progression were evaluated in the present study. Asoxime (HI-6; see figure 1) belongs to potent drugs, the so-called oxime reactivators, that are employed for reactivation of acetylcholinesterase (AChE) in cases of inhibition by nerve agents 15 and it was recognized to suppress some side effects of drugs as tested for cytostatic agent irinotecan. 16 On the other hand, oxime reactivators including asoxime are suspected to modify functional properties of the nicotinic (nAChR) as well as muscarinic (mAChR) acetylcholine receptors. 17-19 The immune system is associated with parasympathetic regulation by the cholinergic anti-inflammatory pathway (CAP) through α7 nAChR localized on macrophages. 20 Pharmacological stimulation of CAP is considered to be beneficial in the septic shock owing to suppression of pro-inflammatory cytokines overproduction. 21 Since asoxime is considered a nAChR inhibitor and its pharmacodynamics allows for only partial penetration through the blood brain barrier22, maximum effects on blood receptors can be expected. In this study, enhancement of macrophages by suppression of CAP by asoxime is a hypothesized way of tularemia progression modulation.

MATERIAL AND METHODS

Microorganism

Francisella tularensis LVS (ATCC 29684) was cryopreserved in liquid nitrogen and subcultured before experiments. Bacterium was cultivated on McLeod agar supplemented with bovine haemoglobin and Iso VitaleX (Becton-Dickinson, San Jose, CA, USA) as described previously. 23 Cells were harvested after two days of cultivation and suspended into saline solution and washed by centrifugation (2,000×g; 10 minutes). The cell concentration was estimated by calibrated cell density meter (WPA, Cam-

Figure 1: *HI-6 structure.*



bridge, UK) and confirmed by a cultivation test two days later.

Animals and blood processing

A total of 70 female BALB/c mice (BioTest, Konarovice, Czech Republic) were divided into 7 groups of 10 animals. Mice were kept in an air conditioned room (22±2°C) with controlled humidity (50±10%) and light (7 a.m to 7 p.m.) within vivarium of the Centre of Biological Defence in Techonin (Czech Republic). Food and water was supplied ad libitum. In the beginning of experiment, mice were eight week old and weighing 24 g. F. tularensis LVS (LVS hereinafter in the text) was adjusted up to 106 CFU/ml. LVS as well as asoxime (Sigma-Aldrich; St.Louis, MO, USA) were suspended in saline solution prior to the application. The groups were following:

1. 100 µl of LVS; 100 µl saline solution
2. 100 µl of LVS; 100 µl asoxime 50 mg/ml (dose 209 mg/kg of body weight)
3. 100 µl of LVS; 100 µl asoxime 5 mg/ml (20.9 mg/kg)
4. 100 µl of LVS; 100 µl asoxime 500 µg/ml (2.09 mg/kg)
5. 100 µl of LVS; 100 µl asoxime 50 µg/ml (209 µg/kg)
6. 100 µl of saline solution; 100 µl asoxime 50 mg/ml (209 mg/kg)
7. 2×100 µl of saline solution

Saline solution, LVS and asoxime solutions were administered subcutaneously in the area of the pelvic limb. Asoxime was administered one hour after LVS. The second round of asoxime, respectively saline solution for groups 1 and 7, application was in the above-mentioned doses on day two of experiment. Mice were sacrificed under CO2 anaesthesia and blood was collected from cervical vessels on day five of experiment. Fresh blood was let to clot for 30 minutes at 4ºC and centrifuged at 500×g for 10 minutes. Freshly prepared serum was used immediately or kept at -80ºC. Spleen and liver were added into phosphate buffered saline: 100 mg tissue per one ml of PBS. Tissues were mixed 8,000 RPM for 1 minute using Ultra-Turrax (Ika Werke, Staufen, Germany). The experiment was repeated with groups 1 - 6 with infection dose 107 CFU/ml. Mice were observed for pertinent mortality until symptomatic manifestation of tularemia become extinct.

Experiments were permitted and supervised by the ethical committee of Centre of Biological Defence (Techonin, Czech Republic) and all responsible personnel were licensed and skilled for the experiments.

Ex vivo assays

Malondialdehyde (MDA), a marker of oxidative stress, was assayed as thiobarbituric acid reactive substance (TBARS) in spleen and liver. The measuring protocol was adopted from the previous experiments. [24] Low molecular weight antioxidants (LMWAs) were assayed as the ferric reducing antioxidant power (FRAP) as described previously [25, 26]. The level of antibodies was estimated using solid phase extraction (SPE) using CBindTM L (Sigma-Aldrich) filled column with subsequent estimation of eluted proteins by the Bradford method according to previous experiments. [23,27-28] Antioxidant properties of asoxime were examined by FRAP method in a way as mentioned above and by quenching of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) using modified experiment according Wozniak et al. [29] Asoxime was tested in concentrations 10-6 - 10-2 M and compared to standard antioxidant Trolox (Sigma-Aldrich). Interleukin 6 (IL6) and interferon γ (IFNγ) were assessed using "Murine IL-6 Eli-pair kit" and "Mouse IFN γ Eli-pair kit" (Abcam, Cambridge, MA, USA) in compliance with provided protocols.

Statistical analysis

Software Origin 8 (OriginLab Corporation, Northampton, MA, USA) was used for data processing. Significance of differences between groups was estimated using one-way analysis of variance with Scheffe test. The significance was recalculated for two probabilities levels P = 0.05 and P = 0.01 for the

Table 1: *Assessment of thiobarbituric acid reactive substances (TBARS) and ferric reducing antioxidant power (FRAP) in organs of experimental animals*

Assay	Organ	Experimental group						
		1	2	3	4	5	6	7
TBARS (μmol/g)	Liver	3.37±0.12	2.27±0.27 (*/-)	2.10±0.28 (*/*)	2.41±0.10	2.53±0.20	2.93±0.22	3.21±0.15
	Spleen	1.83±0.04 (*)	1.48±0.10 (**/-)	1.49±0.14 (**/-)	1.36±0.20 (**/*)	1.48±0.11 (**/-)	1.37±0.14 (**/*)	1.59±0.13
FRAP (μmol/g)	Liver	4.13±0.11 (*)	3.12±0.07 (*/-)	3.32±0.12 (*/-)	3.51±0.10 (*/-)	3.77±0.08	3.09±0.11 (*/-)	3.19±0.16 (*)
	Spleen	2.52±0.05 (*)	2.64±0.15	2.56±0.06	2.58±0.21	2.68±0.11	2.87±0.10 (*/-)	3.21±0.15

Error indicates standard error of mean. One and two asterisks represent significance (ANOVA with Scheffe test) at probability level P = 0.05 (*) and P = 0.01 (**) against group 1 (numerator), respectively group 7 (denominator). Asterisks at group 1 indicate significance to control (group 7).

group size of eight specimens.

RESULTS

In the first round, MDA levels in spleen and liver as well as spleen and liver antioxidant potency by FRAP (table 1) were estimated. Infected mice were compared with the healthy unexposed to asoxime one (group 7). MDA in group 1 was found significantly increased in spleen ($0.01<P \leq 0.05$) but not in liver against the control group 7. The groups 2, 3, 4 and 5 suffering from tularemia were exposed to asoxime in doses 209 μg/kg - 209 mg/kg and they had significantly ($0.01<P \leq 0.05$) decreased MDA level in spleen when compared to the group 1. Livers were significantly ($P > 0.05$) influenced only in animals exposed to the two upper doses of asoxime.

Table 1

Total level of LMWAs indicated by FRAP values were the second examined parameter. We recognized significant ($P > 0.05$) depletion of antioxidants in spleen and increasing in liver in animals infected with tularemia when compared to the controls. Asoxime administration had insignificant impact on FRAP value in spleen but livers of animals in groups 2 - 4 had decreased FRAP to the value insignificant to controls.

Table 2

Total antibodies, IFNγ and IL6 were assessed as selected immunochemical markers. The achieved data are clearly summarized as table 2. The level of antibodies in the group 1 was 12.5±0.5 mg/ml. All mice infected with tularemia had significantly ($P \leq 0.01$) increased antibodies levels when compared with the control group 7. The antibodies ranged from 15.5 ±0.5 (group 1) to 17.2±0.3 mg/ml. Although there was not found a significant difference between antibodies

Table 2: *Plasma immunochemical markers*

Marker	Experimental group						
	1	2	3	4	5	6	7
Ig (g/l)	15.5±0.4 (*)	16.1±0.4 (-/*)	16.3±0.2 (-/*)	16.3±0.3 (-/*)	17.2±0.2 (*/*)	11.8±0.3 (*/-)	12.5±0.3
IFNγ (ng/l)	159±14 (**)	144±8 (-/*)	142±9 (-/*)	149±11 (-/*)	143±8 (-/*)	2.83±0.27 (**/-)	2.58±0.78
IL6 (ng/l)	67.7±2.6 (**)	63.9±6.3 (-/*)	56.7±6.4 (-/*)	49.9±3.5 (-/*)	46.7±2.0 (*/*)	2.83±1.1 (**/-)	2.58±2.2

Error indicates standard error of mean. One and two asterisks represent significance (ANOVA with Scheffe test) at probability level P = 0.05 (*) and P = 0.01 (**) against group 1 (numerator), respectively group 7 (denominator). Asterisks at group 1 indicate significance to control (group 7).

levels in group 1 and groups 2-4, the group 5 challenged with asoxime 209 μg/kg has significantly ($P \leq 0.01$) increased antibodies against the group 1. Asoxime administered to healthy animals (group 6) had no significant effect when compared with the control group 7. Alterations in IFNγ and IL6 had similar tendencies. The both markers were significantly ($0.01<P \leq 0.05$) increased in group 1 compared to the controls. Application of asoxime to the tularemia infected animals caused slight decrease of IFNγ and IL6 levels. The decrease was significant only for IL 6 and asoxime dose 50 μg/ml.

Mortality experiment (cf. figure 2) was carried out in six groups of animals during eleven days. In compliance with expectations, the dose 209 mg/kg in the group 6 caused no death. The asoxime untreated mice infected with tularemia died in a half (52 %). The tularemia infected mice treated with asoxime had significantly decreased mortality ($0.01<P \leq 0.05$; 2x2 contingency table) when compared to the group 1. The group 2 had mortality: 27 %; group 3 and 4: 20 %; group 5: 23 %. The best protective index for asoxime doses 2.09 and 20.9 mg/kg was found: 2.6.

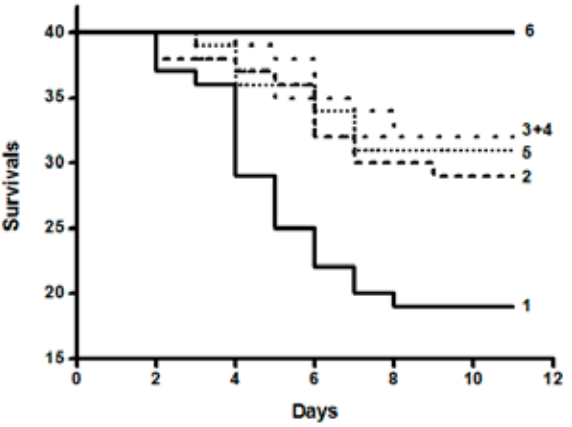
Figure 2

Tests (FRAP and DPPH) of asoxime antioxidant properties did not proved any antoxidant potency in the selected concentration range 10-6 - 10-2 M. No significant increasing of antioxidant properties were found when compared to the saline solution. Functionality of tests (positive control) was examined using standard antioxidant Trolox.

DISCUSSION

Regarding to the previous experiments, the development of tularemia in mice infected by a wild F. tularensis susp. holarctica strain was accompanied with multiple biochemical responses to the growing bacterial burden in tissues and blood [30] such as hypertriglyceridemia, hypercholesterolemia, increasing

Figure 2: *Estimation of mice mortality time response to tularemia and asoxime. Number on the right side indicates experimental group. Line 2 - short dash; line 3 - dot; line 4 - dot; line 5 - short dot.*



liver enzymes and decreasing LMWAs. [31] In this experiment, a less virulent LVS strain was used in this study and blood was collected from mice infected with a sub-lethal dose. The increased FRAP value in livers of mice suffering from tularemia can be attributed to spontaneous protection against oxidative stress and decreased FRAP level in spleen of tularemia infected mice to the uncovered stress. [32] LMWA can be increased in serum due to hyperuricemia; however, it is not typical for tularemia in an extensive scale. [30] The MDA level appoints at oxidative stress burden probably caused by pyroptosis of infected macrophages and consequent release of reactive oxygen species.[33,34] It should be concisely mentioned that F. tularensis is widely resistant to oxidative stress within the phagosome by induction of protective enzymes such as superoxide dismutase. [35] Significant reduction of the MDA value in spleen is the most interesting finding and it could be correlated to the reduced mortality of infected mice. However, it is not clear whether the reduction is caused by reduced pyroptosis or by another regulative mechanism. The asoxime impact was more specific to spleen as the livers were not extensively influenced by asoxime. It is noteworthy to say that MDA level in liver nearly two times higher in all tested animals including control. It refers to fact, that MDA is produced in body due to oxidative phosphorylation and other basic physiological pathways. [36] In liver, the FRAP level in infected animals does not clearly correspond with asoxime administration. The opposite situation was found in spleen. Tularemia infected animals had exhausted LMWAs level in spleen. Lower doses of asoxime caused shift of LMWA that were closer to the value in health animals. It is not clear whether the improved redox balance was caused by suppression of pyroptosis or enhancing of antioxidant potency. When considered overall redox situation in liver and spleen, liver were not exposed to the extensive oxidative stress due to tularemia. FRAP method and DPPH tests excluded asoxime impact as an antioxidant so rather regulative impact, as further discussed bellow, should be expected.

F. tularensis proliferates within macrophages despite the presence of reactive forms of oxygen. [37] T lymphocytes are necessary for enhancing the immune system. Moreover, the lipopolysaccharide (LPS) from F. tularensis does not trigger inflammatory response so the innate immunity is of lower efficacy. [9] Due to this fact, stimulation of the innate immunity could be beneficial for the early phase sustained tularemia. IL17 is necessary for the activation of T lymphocytes and in this way it helps macrophages to kill phagocyted F. tularensis. [38] Restricted growth of F. tularensis in macrophages is also expected due to IFNγ[39]. Pharmacological administration of IFNγ or TNFα can be beneficial for tularemia progress resolving. [40] In a similar way, toll like receptor 3 stimulated with synthetic ligand polyinosine - polycytosine can protect from tularemia pathogenesis as demonstrated on pulmonary tularemia in BALB/c mice. [41] We found no significant alteration in IFNγ after asoxime application. IL6 was influenced more extensively than IFNγ. IL6 was decreasing in a reciprocal proportionality to the asoxime dose. Immunoglobulin levels were influenced in a similar way as IL6: they increased in a reciprocal mode to the asoxime dose. Emphasising the fact that the markers were assessed five days post infection, it seems that asoxime has potency to accelerate specific immune response to the innate one. Improvement of mortality confirms benefits of asoxime application into tularemia infected organisms and practical importance of these findings can be expected.

Our hypothesis is that there is competitive protection of α7 nAChR from stimulation through acetylcholine which enhances immunity and elevates the survival probability during tularemia. However, it is probable that asoxime has ambivalent effects in comparison with other compounds such as neostigmine which modulate CAP by inhibition of AChE only and cause accumulation of acetylcholine in blood[42]. Another drug

with a similar pathway as neostigmine, i.e. the cholinergic agonist physostigmine, was found potent in reducing the oxidative stress and pro-inflammatory chemokines such as high mobility group box 1 (HMGB1). [43] Our recent and still unpublished investigation proposed that asoxime is a non-competitive inhibitor of AChE with a dissociation constant ~10^{-4} mol/l[44]. The interaction of asoxime with AChR is not so clearly understandable and it is the subject of current research. Some studies proposed recently an antagonist effect of pyrimidiumoximes such as obidoxime on mAChR. [45] An effect resulting in acetylcholine release was also expected for pyridinium oximes; however, it was not proven for asoxime. [46] We suppose that positive effects of asoxime administered in low doses prevail over antagonist effects on nAChR to AChE inhibition. A synchronous effect composed from inhibition of AChE and antagonizing of acetylcholine on nAChR is expected in the highest doses of asoxime. The found reciprocal correlations of asoxime dose to immune system confirm this idea. Toxicity of asoxime can also play a role when applied the upper doses.

The achieved data appoint at asoxime potency to modulate the immune system response and to enhance the specific immunity. The achieved amelioration of deaths rate confirms the idea. The results are also in compliance with the previously published paper[47]. We hypothesize that antagonising effect of asoxime on nAChE in blood can accelerate immune response and maturation of cells participating in adaptive immunity via CAP. However, more detailed experiments should be carried out in order to understand link between innate and adaptive immune response modulation by asoxime.

In a conclusion, asoxime was found to be able to ameliorate tularemia progression with protective index 2.6. We found alteration in redox balance in tularemia targeted organs and immune system relation to asoxime impact. Asoxime is considered perspective for practical application in tularemia infected individuals. The next research should elucidate asoxime molecular effects.

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